Natural Antimicrobials in Pregnancy

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Abstract

Natural antimicrobials are peptides that are essential components of the innate immune system, providing broad-spectrum protection against bacteria, yeasts and some viruses. In addition to their innate immune activity, they exhibit properties suggesting they interact with the adaptive immune system. These functions imply they may be of particular importance in pregnancy. Intrauterine infection is responsible for approximately one third of cases of preterm labour, and normal labour is considered an inflammatory process, associated with leukocyte invasion of the uterine tissues and increased cytokine production. Little is known, however, about natural antimicrobial expression in pregnant reproductive tract. The aim of this thesis was thus to characterize natural antimicrobial production in pregnancy. The study focused on two main areas – the lower genital tract, comprised of the vagina and cervix; and the innermost fetal membrane, the amnion.

In the lower genital tract, levels of natural antimicrobials were determined in samples of cervicovaginal secretions collected from pregnant women, using enzyme linked immunosorbance assay (ELISA). In addition Taqman quantitative PCR and ELISAs were used to investigate natural antimicrobial production by cell lines derived from endocervical, ectocervical and vaginal epithelium. It was found that elafin and secretory leucocyte protease inhibitor (SLPI) were found at high concentrations in cervicovaginal secretions, but levels were diminished in women with the common vaginal infection bacterial vaginosis ($p<0.05$). Cells derived from the vaginal epithelium expressed greater amounts of elafin than cervically derived cells. However, elafin and SLPI production could be stimulated in endocervical cells by the bacterial product lipopolysaccharide, a response that was not seen in the vaginal cell line.

Natural antimicrobial production in the amnion was examined in tissue explants and primary cultured amnion cells, using a combination of Taqman PCR and ELISAs. In addition, cDNA microarray was carried out to investigate factors controlling amniotic antimicrobial production, and the involvement of signalling pathways was

studied using specific pathway inhibitors. It was shown that the amnion expressed five antimicrobials: human beta defensins (HBD) 1, 2 and 3, SLPI and elafin. Expression of HBD2 was significantly upregulated following normal labour ($p<0.05$), with production in primary amnion epithelial cells dramatically increased by IL-1ß. The pattern of HBD2 expression in response to IL-1ß was biphasic, which suggested involvement of a secondary gene product. Several putative influential factors were identified by cDNA micorarray, including the NF-kappaB cofactor NF-kappaBinhibitorζ. Its relationship to HBD2 was explored. The involvement of both NF-kappaB and mitogen activated protein (MAP) kinase p38 signalling appeared crucial in the response.

This work has shown that natural antimicrobials are expressed by both the lower genital tract, where infections that are associated with preterm labour originate, and in the amnion, which is the fetus' last line of defence to infection. They may have an important role in the prevention of infection associated preterm labour. Further characterization of these responses may increase understanding of the physiology, and pathophysiology of labour, and lead to strategies for the prevention of premature delivery.

Declaration

Except where due acknowledgment is made by reference the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

Chapter 2

I acknowledge the assistance of Dr Elena Facenda who designed the majority of the primer/probe sets for Taqman quantitative PCR. I acknowledge the assistance of Professor Hilary Critchley, who provided the three endometrial RNA samples for use as positive controls.

Chapter 3

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Chapter 5

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Publications, Presentations and Posters Relating to this Thesis

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ORAL PRESENTATIONS

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POSTERS

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MANUSCRIPTS IN PREPARATION

Levels of Elafin are Diminished in Cervicovaginal Secretions in Association with Bacterial Vaginosis

The effect of IL-1β on the amniotic epithelium

Contents

Chapter 3: NATURAL ANTIMICROBIAL PRODUCTION BY THE CERVIX AND VAGINA

Chapter 4: NATURAL ANTIMICROBIAL EXPRESSION IN THE AMNION

Chapter 5: THE EFFECT OF IL-1B ON THE AMNIOTIC EPITHELIUM

List of Figures

Chapter 1: LITERATURE REVIEW

:

Chapter 2: GENERAL METHODS

Chapter 3: NATURAL ANTIMICROBIAL PRODUCTION BY THE CERVIX AND VAGINA

Appendix 4

Abbreviations

ABC	Avidin Biotin Complex Peroxidase Detection System
ANOVA	Analysis of Variance
AP-1	Activator protein-1
ATCC	American Type Culture Collection
ATF	Activating Transcription Factor
BLAST	Basic Local Alignment Tool
BMI	Body Mass Index
BMP2	Bone Morphogenic Protein 2
BPE	Bovine Pituitary Extract
BPI	Bacterial Permeability Inducing Factor
BSA	Bovine Serum Albumin
BV	Bacterial Vaginosis
C/EBP	CAAT/Enhancer-Binding Protein
CCL	C-C Motif Ligand
CCLR	C-C Motif Ligand Receptor
CD	Cluster Determinant
cDNA	Complementary DNA
CEMACH	Confidential enquiry into maternal and child health
CI	Confidence Interval
COX-1/2	Cyclo-Oxygenase 1/ 2 (PTGS 1/2)
CRH	Corticotrophin Releasing Hormone
CT	Cycle Threshold
CXCL	C-X-C Motif Ligand
DAB	3,3 -diaminobenzidine
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
ds RNA	Double stranded RNA
ECT	Ectocervical
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbance Assay
END	Endocervical
ERK	Extracellular Signal Regulated Kinase
ET-1	Endothelin 1
ETS	Erythroblast Transformation Specific Domain
FAM	6-Carboxyfluorescein
FCS	Fetal Calf Serum
FISH	Fluorescent *In Situ* Hybridization

G-CSF	Granulocyte Colony-Stimulating Factor
GITC	Guanidine thiocyanate
GO	Gene Ontology
GROα/β/γ	Growth Related Oncogene alpha/beta/gamma
H+E	Haematoxylin and Eosin
H_2O	Water
H_2O_2	Hydrogen Peroxide
HBD	Human Beta Defensin
HD	Human Defensin
HIV	Human Immunodeficiency Virus
HNP	Human Neutrophil Peptide
IFNγ	Interferon gamma
IκB α/β/γ	Inhibitor of Kappa B α/β/γ
IKKα/β/γ	IκB kinase α/β/γ
IL-1,6,8,17 etc	Interleukin 1, 6, 8, 17 etc
IL-17R	Interleukin 17 receptor
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-1RA	Interleukin 1 Receptor Antagonist
IL-1-R-AcP	Interleukin 1 Receptor Accessory Protein
IL-1RI/II	Interleukin receptor type I/II
IRAK	IL-1 Receptor Associated Kinase
kDa	Kilo Dalton
LBP	Lipopolysaccharide Binding Protein
LMP	Last Menstrual Period
LPS	Lipopolysaccharide
LSCS	Lower Segment Caesarean Section
LTA	Lipoteichoic acid
MAL	MYD88 Activator Like Protein
MAPK	Mitogen Activated Protein Kinase
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MEKK1/2/3	MAPK/ERK Kinase Kinase 1/2/3
$MgCl_2$	Magnesium Chloride
MIAME	Minimum Information about a Microarray Experiment
MIP1α/β	Macrophage Inflammatory Protein 1 α/β
MKK	MAPK-kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MUC1	Mucin 1
MYD88	Myeloid Differentiation Primary Response Gene 88
NBF	Neutral Buffered Formalin

NEMO	NFκB Essential Modulator
NFκB	Nuclear Factor Kappa B
NFκBI	NFκB inhibitor (gene coding for IκB)
NIK	NCK Interacting Kinase
NK	Natural Killer
NLS	Nuclear Localization Signal
NRF	NFκB Repressing Factor
NRS	Normal Rabbit Serum
OCT	Octamer Binding Protein
OD	Optical Density
OR	Odds ratio
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGDH	Prostaglandin Dehydrogenase
PGH_2	Prostaglandin H2
PPROM	Preterm Prelabour Rupture of Membranes
PTGS	Prostaglandin-Endoperoxidase Synthase (COX2)
RANTES	Regulated on Activation, Normal T-Cell Expressed and Secreted
REC	Research Ethics Committee
RHD	Rel homology domain
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute
rsd	Relative Standard Deviation
RT	Reverse Transcription
SEM	Standard Error of the Mean
SLPI	Secretory Leukocyte Protease Inhibitor
spp	Species
SRE	Serum response element
ss RNA	Single Stranded RNA
STAT	Signal Transducer and Activator of Transcription
SVD	Spontaneous Vertex Delivery
TAK-1	TGFβ activated kinase-1
TAMRA	6- carboxytetramethylrhodamine
TE	Tris/EDTA buffer
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Matrix Metalloproteinases
TIR	Toll-like/Interleukin Receptor

TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor Alpha
TNFAIP3	TNF Alpha Induce Protein 3
TNFR	Tumour Necrosis Factor Alpha Receptor
TOLLIP	Toll Interacting Protein
TRADD	TNF-Receptor-Death Domain
TRAF	TNF Receptor-Associated Factors
TRAM	TRIF-Related Activator Molecule
TRIF	TIR Domain Containing Adaptor Inducing Interferon-ß
VIC	Chemical Name Not Released (Proprietary to ABI)
VK	Vaginal Keratinocyte

1. Literature Review

1.1. INTRODUCTION

Parturition is characterized by profound changes in uterine and extrauterine physiology, culminating in increased myometrial contractility and cervical effacement and dilation. The mechanisms controlling the onset of labour are incompletely understood. Nevertheless, decidual and fetal membrane activation and inflammation of the gestational tissues are key features of the final common pathway leading to delivery. Tight regulation of these processes is essential, as an untimely, uncontrolled or inadequate response can lead to preterm labour.

Intrauterine infection is a major cause of preterm labour, and appears to be particularly associated with early preterm birth. It can also cause activation of the fetal inflammatory response, increasing severe neonatal morbidities in these high risk infants. Natural antimicrobials are a family of multifunctional proteins produced by epithelial and inflammatory cells which have broad-spectrum activity against bacteria. They also can modulate the immune response, and their involvement in the pathophysiology of a number of infective and inflammatory conditions is recognized. Little is known about natural antimicrobials in pregnancy, but they could be important components linking infection, inflammation, and labour.

This thesis examines the production of epithelial natural antimicrobials in pregnancy. Evidence that natural antimicrobials are expressed in gestational tissues is provided. In addition, their involvement in the process of parturition is explored and mechanisms regulating their secretion investigated. Further research areas are highlighted and potential roles in the prevention of infection and preterm labour speculated upon.

1.2. THE REPRODUCTIVE TRACT IN PREGNANCY

1.2.1. The Uterus

The body of the uterus is predominantly comprised of bundles of smooth muscle joined by connective tissue containing many elastic fibres. During pregnancy it undergoes remarkable growth. The non-pregnant uterine cavity has a volume of around ten millilitres; at term the average volume is five litres. This enlargement is mainly secondary to hypertrophy of the muscle fibres, along with an accumulation of fibrous tissue and elastic tissue. There are also increases in blood vessels and lymphatics. In the first trimester uterine growth is driven by oestrogen, and possibly progesterone. After twelve weeks the expanding uterine contents exert the greater effect on the increase in uterine size. By term the uterus has changed from a firm, thick walled structure, to a thin musculo-elastic sac.

The uterus undergoes spontaneous, mild contractions from the first trimester onwards (Braxton-Hicks contractions) which are initially irregular, painless and infrequent. Towards the end of gestation these become more frequent and may develop some pattern (Challis, Lye et al. 2001) This is associated with an increase in responsiveness to uterotonins, mediated by dramatic increases in the number of oxytocin-receptors (Ivell, Kimura et al. 2001) and connexins genes (gap junctions) allowing increased coupling between myometrial cells (Chow and Lye 1994; Lefebvre, Piersanti et al. 1995). A number of other pro-labour or contraction-associated genes are upregulated, including prostaglandin synthetic enzymes (Challis, Sloboda et al. 2002) which promote the establishment of co-ordinated contractions leading to parturition.

The non-pregnant uterine cavity is lined by endometrium, which in pregnancy becomes decidualized early in the process of implantation, at about day 7-10 post-conception. The area invaded by the blastocyst is the decidua basalis, wheras the decidua capsularis covers the implantation site, and the decidua parietalis is that which is away from the implantation site. The functions of decidua are not fully

understood, but its crucial position at the maternal-fetal interface suggests it has functions in communicating between the mother and fetus. It may have roles in early pregnancy development, regulation of the invading trophoblast into the maternal tissue and mediation of immune events during pregnancy. It is a metabolically active tissue, and can secrete factors which may influence the fetal tissues, as well as being responsive to mediators produced by them.

1.2.2. The Cervix

The cervix is a cylindrical structure which is continuous with the body of the uterus. It is approximately four centimeters long by one centimeter wide in the non-pregnant state, and grows slightly in pregnancy. Its inner surface is called the endocervix, and is lined with columnar epithelium containing many branching glands. The ectocervix is the portion which protrudes into the vagina, and this is covered by stratified squamous epithelium. The two types of epithelia meet at an area called the transition zone, around the level of the external os. Underlying the epithelia is connective tissue, made up of collagens (mainly type I and III) embedded in proteoglycans. There is also a cellular component made up of fibroblasts and smooth muscle cells (Norman, Thomson et al, 2004).

The cervix dilates during labour to a maximum diameter of ten centimetres, to allow delivery of the fetus from the uterus. Cervical ripening is the process by which the cervix changes from a firm barrier retaining the pregnancy, to a pliable organ which yields to the passage of the fetus during parturition. Ripening precedes the onset of contractions by some weeks, and is mediated by chemokines and prostaglandins (Kelly 2002). These induce neutrophil infiltration of the cervix, which produce the collagenases and matrix metalloproteinases that remodel the cervical extracellular matrix (Osman, Young et al. 2003). Hydrophobic glycosaminoglycans within the connective tissue are replaced by more hydrophilic hyaluronic acid, and the water content of the cervix is increased, whilst its collagen content decreases.

1.2.3. The Fetal Membranes

The developing human fetus is surrounded by amniotic fluid contained in extra-embryonic tissues known collectively as the fetal membranes. The inner layer, the amnion, contains the amniotic fluid, whilst the outer chorion laeve lies adjacent to the maternal decidua.

1.2.3.1. *Structure of the Fetal Membranes*

Amnion

The amnion is first identifiable around day 7-8 after fertilization, before gastrulation. It develops as a space that develops in the inner cell mass adjacent to the trophoblast. The cells that line this are called amnioblasts, and are the precursors of amnion epithelial cells. They are derived from epiblasts, which are the cells which also give rise to the three germ cell layers. The amniotic epithelium retains some of the multipotency of epiblast cells, right up to term (Miki, Lehmann et al. 2005).

Early in embryogenesis the amnion is composed of an inner layer of amnion epithelial cells immediately adjacent to a layer of mesenchymal cells. As the amniotic sac expands, the cuboidal epithelial cells replicate at a sufficient rate to maintain a continuous layer of cells, connected by desmosomes. This secretes collagen types III and IV and the non-collagenous glycoproteins (laminin, nidogen and fibronectin) which form a basement membrane for the cells. The majority of the tensile strength of the fetal membranes is provided by the *zona compacta*, synthesized by the mesenchymal cells (Casey and MacDonald 1996). This is comprised of parallel bundles of interstitial collagens (type I and III) linked to the basement membrane by filamentous connections of collagen V and VI (Malak, Ockleford et al. 1993). Division of the mesenchymal cells apparently does not keep pace with the growing amniotic sac, and they start to become dispersed from around 10-14 weeks. By the third trimester there are only around one-tenth as many mesenchymal cells as epithelial cells, connected by a loose network of connective tissue. The spongy or intermediate layer lies between the amnion and chorion and contains a non-fibrillar network of collagen (type III) with abundant hydrated

proteoglycans and glycoproteins (Malak, Ockleford et al. 1993). There are a few fetal macrophages in the amnion, but it is entirely devoid of vasculature, lymphatics, smooth muscle and nerve. It is nourished by the amniotic fluid and by simple diffusion of nutrients from the chorionic vessels. (Bourne 1962; Bryant-Greenwood 1998)

Chorion

The chorion is approximately 4 times the thickness of the amnion but has far less tensile strength. It comprises a basal and a superficial population of cells. The basal cells are tightly packed cytotrophoblast cells, adjacent to a basement membrane. The superficial cells are mainly fibroblast which are more dispersed, and stabilized by an underlying reticular layer. On the outermost surface are remnants of the villous stroma adhering to the maternal decidua. In contrast to the amnion, the chorion is vascularized. Chorionic mesoderm, particularly at the fetal side, contains vessels throughout the second trimester and the maternal side has vessels until term (Bernirschke and Kaufmann, 1995).

1.2.3.2. *Functions of the Fetal Membranes*

Mechanical Protection

The amniotic fluid cushions the fetus and helps maintain temperature. It aids musculoskeletal development, and is essential for normal pulmonary and gastrointestinal development (Underwood, Gilbert et al. 2005). The fetal membranes have sufficient strength and elasticity to progressively stretch to approximately double their size by term, whilst simultaneously protecting against rapid pressure changes caused by fetal movements or external pressure to the maternal abdomen (Bryant-Greenwood 1998).

Antimicrobial Properties

The amniotic cavity resists penetration by potentially harmful microbes, leukocytes and neoplastic cells from the maternal compartment. The antimicrobial activity of amniotic fluid has long been recognised (Miller, Michel et al. 1976; Tafari, Ross et al. 1977; Sachs and Stern 1979; Thomas, Sbarra et al. 1988). Moreover, host defence

proteins such as lipopolysaccahride binding protein (LBP) and CD14 which are crucial for bacterial recognition and elimination, are essential components of amniotic fluid (Roos, Martin et al. 1997; Gardella, Hitti et al. 2001; Espinoza, Romero et al. 2002).

In vitro both amnion and chorion have an inhibitory effect on the growth of a diverse range of bacteria, and chorioamnion provide a competent barrier to penetration by Group B Streptococcus (Kjaergaard, Helmig et al. 1999; Kjaergaard, Hein et al. 2001). *In vivo* chorioamniotic membrane has been used as an effective antimicrobial dressing for burns and ulcerated skin and cornea, reducing inflammation and promoting repair (Ravishanker, Bath et al. 2003; Gomes, Romano et al. 2005). Profiling of gene expression patterns in the placenta has shown that the amnion expresses high levels of genes such as *MUC 1,* which codes for a glycoprotein that could confer antibacterial and antiadhesive properties to the amnion (Sood, Zehnder et al. 2006).

Paracrine interactions and membrane degradation

The fetal membranes form a bidirectional paracrine link between mother and fetus. Fetal – maternal communication occurs via amniotic fluid constituents such as fetal urine and lung secretions, whilst in the other direction maternal decidual and blood products can pass into the amniotic fluid and enter the fetus via fetal breathing and swallowing. In addition, the fetal membranes themselves are metabolically active tissues, capable of producing a variety of bioactive compounds. These may act locally in the gestational tissues and uterus, but also pass into the amniotic fluid to gain access to the fetus, and are involved in a diverse array of physiological processes.

It is critical that the membranes are receptive to signals from the fetus, mother and fetal-maternal interface, in order to co-ordinate the process of degradation which is essential for parturition. A cascade of events occurs involving membrane distortion, extracellular matrix deformation and loss of cell-matrix interactions, apoptosis, (Menon, Lombardi et al. 2002; Fortunato and Menon 2003) matrix metalloproteinase

(MMP)-activation (Bryant-Greenwood and Yamamoto 1995; Riley, Leask et al. 1999; Menon and Fortunato 2004) and membrane degradation (Lei, Furth et al. 1996). The timing of this response is crucial, as prelabour preterm rupture of the membranes is associated with approximately 30% of cases of preterm delivery (Bryant-Greenwood and Millar 2000; Arias and Tomich 1982).

1.2.4. The Vagina

The vagina is a tubular musculo-membranous structure that extends from the uterus to the vulva. The upper third arises from the mullerian duct, along with the uterus and cervix, whereas the lower third arises from the urogenital sinus. The vagina is separated from its relations, anteriorly the bladder and urethra, and posteriorly the rectum, by connective tissue called the vesicovaginal septum and rectovaginal septum. The upper portion of the vagina is also usually separated from the rectum by the pouch of Douglas. The upper end of the vaginal vault is subdivided into the anterior, posterior and two lateral fornices by the protruding uterine cervix. The vaginal wall is attached to the posterior wall of the cervix at a higher level than the anterior wall, thus the posterior fornix is deeper.

The mucosa of the vagina has multiple transverse folds or *rugae*, and is lined by non-keratinized stratified squamous epithelium. It is devoid of glands, but is lubricated by mucus from the cervix, secretions from the uterus and vestibular glands, and a transudate from the rich vascular network of the lamina propria. The mucosa is influenced by the menstrual cycle, and under the influence of circulating oestrogens in the follicular phase, the epithelial cells synthesize and accumulate glycogen. When intact, the mucosal barrier of the vagina forms a physical barrier to infection, and cells are continuously desquamated, eliminating attached microbes. The integrity of the mucosal barrier can be affected by bacterial enzymes allowing penetration of pathogens (Cauci, Driussi et al. 1998; Olmsted, Meyn et al. 2003).

Lactobacilli are the facultative anaerobic Gram-positive bacteria normally populating the vagina, which provide important protection against vaginal infection. They utilise

nutrients and produce lactic acid from glucose, which maintains an inhospitable low pH in the vagina. At low pH they produce bacteriocin-like substances which are bacteriostatic, and biosurfactants that help prevent adherence of pathogens to the vaginal epithelium.

1.3. THE INNATE IMMUNE RESPONSE AND INFLAMMATION

The immune system is comprised of two interacting arms- the innate immune system and the adaptive immune system (Figure 1.1). The innate immune system is phytogenetically ancient and provides the first line of defence against infection. It is characteristically rapid and non-specific, preventing penetration of pathogens and effecting microbial killing until recruitment of the slower, but more specific adaptive immune response (Medzhitov and Janeway 2000). Epithelial cells, granulocytes and macrophages are the principal cell types of the innate immune response, whereas the adaptive immune response is provided by cells of the lymphoid lineage.

The innate immune response is responsible for stereotypical inflammation seen in response to tissue damage, which can occur as a result of physical injury or physiological processes such as ovulation and menstruation. Many components of the innate immune response are genetically determined resulting in substantial variation in the inflammatory response between individuals (Imahara and O'Keefe 2004). Tissues which interface the external environment, and as such are exposed to pathogens, are crucial components of the innate immune system. Epithelial cells form a physical barrier to microorganisms, but also actively contribute to microbial elimination via production of natural antimicrobials and opsonins. Upon physical damage or bacterial penetration, they can produce a variety of inflammatory mediators such as chemokines, cytokines and prostaglandins. These augment local blood flow and enable the recruitment of leukocytes, which reinforce inflammatory mediator production and signal to the adaptive immune system (Dempsey, Vaidya et al. 2003). Incoming and resident phagocytes can remove cellular debris, and vascular capillary endothelial and fibroblast proliferation result in granulation tissue formation facilitating tissue repair. Systemic features, such as fever and the acute

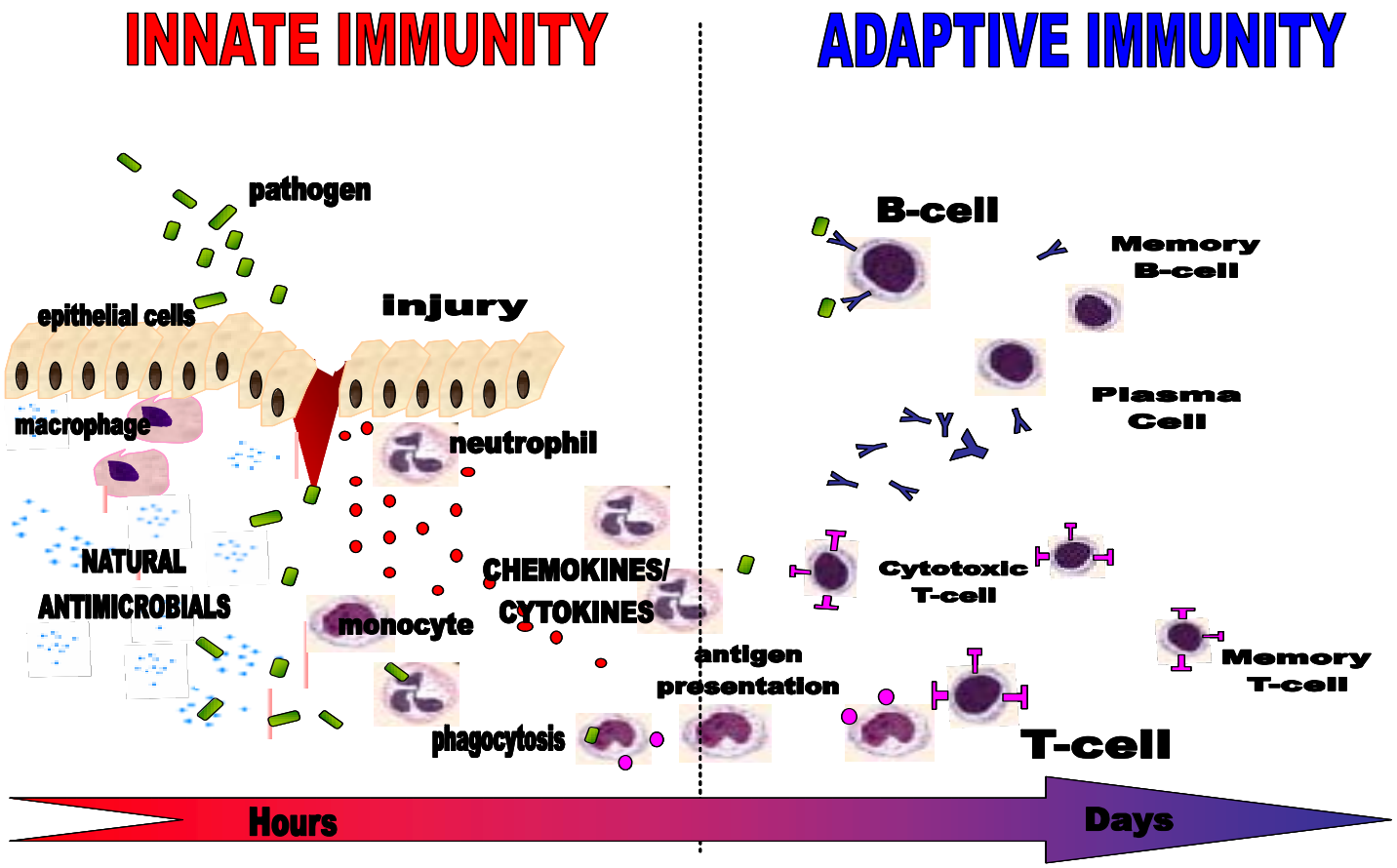


Figure 1.1

Diagramatic representation of the innate and adaptive arms of the immune system.

phase response are also sometimes a feature. Tight control of inflammation is essential, to ensure successful removal/repair of the injurious agent, without excessive tissue damage. This involves regulatory factors including glucocorticoids and anti-inflammatory cytokines, as well as other unknown mechanisms.

1.3.1. Mediators of Inflammation

The inflammatory response is mediated by a plethora cytokines, chemokines and enzymes (Matsukawa, Hogaboam et al. 2000). Many have common functional activities, and redundancy is high. Examples of mediators with particular relevance to this thesis and/or pregnancy are highlighted below.

1.3.1.1. *Cytokines*

Cytokines are soluble proteins or glycoproteins that act as mediators of intra- and intercellular communication, and are involved in processes of development, tissue repair, haematopoiesis, inflammation and the immune response (Hibbert and Johnston 2001). Cytokines function in a complex network where one cytokine can influence production of, and response to, many other cytokines. They act at nano to pico molar concentrations, interacting with cell surface receptors coupled to intracellular signalling systems and second messenger pathways to effect their action (Haddad 2002).

Interleukin-1 (IL-1), Tumour Necrosis Factor Alpha (TNFα) and Interleukin-17 (IL-17) are key cytokines involved in the inflammatory response.

IL-1

IL-1 is a multifunctional pro-inflammatory cytokine. It exists in two forms- IL-1α and IL-1β, which are products of separate genes. These are 17kDa proteins consisting of 159 amino acids and 153 amino acids respectively. They exhibit only 27% protein sequence homology, but are nearly identical in 3 dimensional structure and their biological effects are generally indistinguishable (www.copewithcytokines.com). Two IL-1 receptors (type 1 [IL-1RI] and 2 [IL-

1RII]) have been described. IL-1α and IL-1β bind to both receptor types, but only IL-1RI transduces a signal, whilst IL-1RII acts as a decoy receptor. An endogenous antagonist (IL-1RA) of IL-1 has also been identified, which competitively binds IL-1RI. Receptors are found almost ubiquitously on somatic cells.

IL-1 is secreted by a multitude of cell types, with macrophages, monocyte and dendritic cells being major sites of production. Its expression may be invoked by a variety of factors including TNFα and endotoxin, whilst inhibitors of secretion include prostaglandin E_2 (PGE_2), glucocorticoids and IL-1RA. It has numerous biological effects including chemotaxis of neutrophils and up-regulation of adhesion molecules, activation of T helper cells and Natural Killer (NK) cells, stimulation of immunoglobulin production, and stimulation of other cytokines. It can act directly or indirectly via the induction of PGE_2, IL-6, and IL-8 and it also synergises with other cytokines such as TNFα (Dinarello 1996).

Studies demonstrating IL-1 expression in the placenta and membranes are detailed in Table 1.1. In addition IL-1 is found in myometrium(Winkler, Fischer et al. 1998), and cervicovaginal secretions in pregnancy (Mattsby-Baltzer, Platz-Christensen et al. 1998; Wennerholm, Holm et al. 1998; Doh, Barton et al. 2004; Torbe and Czajka 2004; Gonzalez Bosquet, Ferrer et al. 2005; Kalinka, Sobala et al. 2005; Culhane, Nyirjesy et al. 2006).

IL-1B	*Protein*	*RNA*
Villous Placenta	(Baergen, Benirschke et al. 1994)[ce] (Benyo, Miles et al. 1997)[ce] (Berkowitz, Faris et al. 1990)[abcd] (Flynn, Finke et al. 1982)[d] (Flynn, Finke et al. 1985)[cd] (Gunn, Hardiman et al. 1996)[c] (Haynes, Jackson et al. 1993)[ae] (Hu, Yang et al. 1992)[ace] (Jokhi, King et al. 1997)[ae] (Kauma, Matt et al. 1990)[a] (Keelan, Marvin et al. 1999)[c] (Kelly, Carr et al. 1995)[a] (Laham, Brennecke et al. 1996)[bc] (Librach, Feigenbaum et al. 1994)[ace] (Paulesu, King et al. 1991)[abce] (Simon, Frances et al. 1994)[ae] (Stallmach, Hebisch et al. 1995)[ce] (Steinborn, Gunes et al. 1996)[c] (Steinborn, von Gall et al. 1998)[c] (Taniguchi, Matsuzaki et al. 1991)[c]	(Ammala, Nyman et al. 1997)[c] (Bennett, Lagoo-Deenadayalan et al. 1996)[ce] (Bennett, Lagoo-Deenadayalan et al. 1998)[a] (Bennett, Lagoo-Deenadayalan et al. 1999)[a] (Haynes, Jackson et al. 1993)[ae] (Heinig, Wilhelm et al. 1993)[c] (Kauma, Matt et al. 1990)[a] (Stephanou, Myatt et al. 1995)[ce]
Decidua	(Baergen, Benirschke et al. 1994)[c] (Gunn, Hardiman et al. 1996)[c] (Jokhi, King et al. 1997)[a] (Kauma, Matt et al. 1990)[a] (Lonsdale, Elder et al. 1996)[a] (Keelan, Marvin et al. 1999)[c] (Paradowska, Blach-Olszewska et al. 1997)[c] (Romero, Wu et al. 1989)[c] (Simon, Frances et al. 1994)[ad] (Steinborn, Gunes et al. 1996)[c] (Young, Thomson et al. 2002)[cd]	(Ammala, Nyman et al. 1997)[c] (Kauma, Matt et al. 1990)[a] (Saito, Nishikawa et al. 1993)[ad]
Fetal Membranes	(Baergen, Benirschke et al. 1994)[cg] (Gunn, Hardiman et al. 1996)[cfg] (Kauma, Matt et al. 1990)[afg] (Keelan, Marvin et al. 1999)[cfg] (Menon, Swan et al. 1995)[cfg] (Paradowska, Blach-Olszewska et al. 1997)[cg] (Young, Thomson et al. 2002)[cd]	(Kauma, Matt et al. 1990)[afg] (Menon, Swan et al. 1995)[cf]

TNFA	*PROTEIN*	*RNA*
Villous Placenta	(Benyo, Miles et al. 1997)[ce] (Berkowitz, Faris et al. 1990)[abcd] (Chen, Yang et al. 1991) [ace] (Haynes, Jackson et al. 1993) [ae] (Jaattela, Kuusela et al. 1988)[c](Jokhi, King et al. 1997)[ae] (Kelly, Carr et al. 1995)[a] (Laham, Brennecke et al. 1994)[bc] (Paradowska, Blach-Olszewska et al. 1997)[c] (Pijnenborg, McLaughlin et al. 1998)[abce] (Stallmach, Hebisch et al. 1995)[ce] (Steinborn, Gunes et al. 1996)[c] (Steinborn, von Gall et al. 1998)[c](Vince, Shorter et al. 1992)[acde]	(Bennett, Lagoo-Deenadayalan et al. 1996) [ce] (Bennett, Lagoo-Deenadayalan et al. 1998) [a] (Bennett, Lagoo-Deenadayalan et al. 1999) [a] (Chen, Yang et al. 1991) [ace] (Haynes, Jackson et al. 1993) [ae] (Heinig, Wilhelm et al. 1993) [c] (King, Jokhi et al. 1995) [ae] (Vince, Shorter et al. 1992) [ac] (Yang, Yelavarthi et al. 1993) [ae]
Decidua	(Chen, Yang et al. 1991) [ac] (Deniz, Christmas et al. 1996) [c] (Jaattela, Kuusela et al. 1988) [c] (Jokhi, King et al. 1997) [a] (Laham, Brennecke et al. 1994)[c] (Lonsdale, Elder et al. 1996) [a] (Paradowska, Blach-Olszewska et al. 1997) [c] (Saito, Kasahara et al. 1993) [ad] (Steinborn, Gunes et al. 1996) [c] (Vince, Shorter et al. 1992)[ac] (Young, Thomson et al. 2002) [cd]	(Chen, Yang et al. 1991) [ac] (Jokhi, King et al. 1997) [ad] (Saito, Kasahara et al. 1993) [ad] (Vince, Shorter et al. 1992) [acd] (Vives, Balasch et al. 1999) [ac]
Fetal Membranes	(Chen, Yang et al. 1991)[cfg] (Fortunato, Menon et al. 1994)[cg] (Paradowska, Blach-Olszewska et al. 1997)[cg] (Young, Thomson et al. 2002)[cdfg]	(Chen, Yang et al. 1991)[cf] (Fortunato, Menon et al. 1994)[cfg]

IL-17	*PROTEIN*	*RNA*
Villous Placenta	(Pongcharoen, Somran et al. 2007) [acde]	

IL-8	*PROTEIN*	*RNA*
Villous Placenta	(Denison, Kelly et al. 1998)[c] (Elliott, Kelly et al. 1998) [ace] (Keelan, Marvin et al. 1999) [c] (Laham, Brennecke et al. 1997) [c] (Laham, Brennecke et al. 1999) [c] (Saito, Kasahara et al. 1994) [acde] (Shimoya, Matsuzaki et al. 1992) [abcde] (Stallmach, Hebisch et al. 1995)[c]	(Saito, Kasahara et al. 1994) [acde] (Shimoya, Matsuzaki et al. 1992) [abc]
Decidua	(Denison, Kelly et al. 1998) [c] (Keelan, Marvin et al. 1999) [c] (Saito, Kasahara et al. 1994) [acd] (Saito, Kasahara et al. 1994) [ad] (Young, Thomson et al. 2002) [cd]	(Dudley, Collmer et al. 1996) [c] (Saito, Kasahara et al. 1994) [ac] (Saito, Kasahara et al. 1994) [ad]
Fetal Membranes	(Denison, Kelly et al. 1998) [cfg] (Dudley, Collmer et al. 1996) [cf] (Fortunato, Menon et al. 1995) [cfg] (Ito, Nakamura et al. 1994) [cf] (Keelan, Sato et al. 1997) [cg] (Keelan, Marvin et al. 1999) [cfg] (Laham, Brennecke et al. 1997) [cg] (Laham, Brennecke et al. 1999) [cg] (Trautman, Dudley et al. 1992) [cg] (Young, Thomson et al. 2002) [cdf]	(Dudley, Collmer et al. 1996) [cfg] (Fortunato, Menon et al. 1995) [cfg]

Table 1.1

Inflammatory mediator expression in placenta and membranes (adapted from (Bowen, Chamley et al. 2002)).

[a]=first trimester; [b]=second trimester; [c]=third trimester; [d]=localized to immune cells within tissue (at least in part); [e]=localized to trophoblast (at least in part); [f]=chorion; [g]=amnion.

TNFα

TNFα is another prototypic inflammatory cytokine, with multiple pro-inflammatory functions. It is a non-glycosylated protein of 17 kDa and has a length of 157 amino acids. High-affinity receptors for TNFα are expressed on all somatic cell types with the exception of erythrocytes (www.copewithcytokines.com).

TNFα is secreted by macrophages, monocytes, granulocytes, T lymphocytes and NK cells following their stimulation by bacterial lipopolysaccharides. Its synthesis is also induced by inflammatory stimuli including interferons and IL-2, whereas TGF-β, prostaglandin E_2 and dexamethasone inhibit its production. TNFα enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and also modulates the expression of many other proteins, including IL-1 and IL-6 and a number of other chemoattractant cytokines. It is a potent chemoattractant for neutrophils and increases their adherence to the endothelium. TNFα is also pro-apoptotic and cytotoxic to malignant cells (Locksley, Killeen et al. 2001).

Studies demonstrating TNFα expression in the placenta and membranes are outlined in Table 1.1. It is also found in cervicovaginal secretions (Mattsby-Baltzer, Platz-Christensen et al. 1998) in pregnancy. In addition it has been immunolocalized in the epithelium of the cervix in labour (Young, Thomson et al. 2002).

IL-17

IL-17 is a disulfide-linked homodimeric glycoprotein consisting of 155 amino acids exerting its actions as a homodimer with a molecular weight around 35 kDa (Yao, Painter et al. 1995). IL-17 is a product of activated $CD4^+$ memory T lymphocytes but $CD8^+$ memory T lymphocytes can also produce IL-17 after stimulation and it has also been detected in eosinophils, neutrophils and human blood monocytes (Kolls and Linden 2004). The IL-17 receptor (IL-17R) is a type I transmembrane protein and can be detected in epithelial cells, fibroblasts, B and T lymphocytes, myelomonocytic cells, marrow stromal cells and vascular endothelial cells (Moseley, Haudenschild et al. 2003). IL-17 appears to be involved in the coordination of local

tissue inflammation via the induced release of proinflammatory and neutrophil-mobilizing cytokines.

In vitro stimulation with IL-17 stimulates production of IL-6, (Yao, Painter et al. 1995), IL-8, CXCL1 and granulocyte colony-stimulating factor (G-CSF) in human bronchial epithelial cells (Jones and Chan 2002), and monocytes release TNFα and IL-1β when stimulated with IL-17 (Jovanovic, Di Battista et al. 1998). In mice, IL-17 is induced in response to Gram-negative bacterial challenge, and mice lacking IL-17R succumb to bacteraemia and early death after exposure to *Klebsiella pneumoniae* (Ye, Garvey et al. 2001).

IL-17 has recently been localized in placental trophoblast, and placental macrophages (Pongcharoen, Somran et al. 2007) but there are no reports of IL-17 or IL-17R expression in the fetal membranes.

1.3.1.2. *Chemokines*

Chemokines are structurally related glycoproteins with chemotactic and/or leukocyte activation capabilities. They are smaller than cytokines- approximately 70 to 130 amino acids in length and approximately 8 to 10 kDa in molecular weight. They are classified into two main subfamilies based on the position of two amino terminal cysteine residues, or a four-cysteine motif. CXC chemokines contain a single amino acid between the first and second cysteine residues and are generally chemoattractant to neutrophils. CC chemokines have adjacent cysteine residues and tend to be involved in recruitment of monocytes, lymphocytes, basophils, and eosinophils. Two variations have also been identified: lymphotactin, which is specific for lymphocytes, and is missing a cysteine at its amnino terminus; and fractalkine which has three amino acid residues between the first two cysteines and induces both adhesion and migration of leukocytes (www.copewithcytokines.com).

Chemokines act via heptahelical G-coupled receptors. Six CXC receptors (CXCR1-6) and ten CC receptors (CCR1-10) have been identified. The majority of receptors recognise more than one chemokine, and some chemokines bind to more than one receptor (Baggiolini 2001).

IL-8

IL-8 (CXCL8) is a potent neutrophil chemokine and an archetypal member of the CXCL family. It is a non-glycosylated protein of 72 amino acids, produced by processing a precursor of 99 amino acids. IL-8 is secreted by many cell types including monocytes, macrophages, fibroblasts, endothelial cells, and epithelial cells, most often after stimulation by IL-1 or TNFα. It signals through CXCR1 and 2 expressed on neutrophils and T lymphocytes (www.copewithcytokines.com).

IL-8 induces rapid changes in neutrophil shape, activation of endothelial adherence factors, and the release of granule contents and formation of oxygen radicals. Granule release occurs at relatively high IL-8 concentrations and is enhanced after treatment of the cells with inflammatory cytokines. This suggests that it may occur once the neutrophil has reached its target, where high levels of chemokines promote antimicrobial activity (Baggiolini 2001).

Studies demonstrating IL-8 expression in the placenta and membranes are shown in Table 1.1. It is also found in cervicovaginal secretions (Mattsby-Baltzer, Platz-Christensen et al. 1998; Wennerholm, Holm et al. 1998; Torbe and Czajka 2004; Diaz-Cueto, Cuica-Flores et al. 2005; Gonzalez Bosquet, Ferrer et al. 2005; Kalinka, Sobala et al. 2005; Culhane, Nyirjesy et al. 2006) in pregnancy and has been immunolocalized in the epithelium and stroma of the cervix in labour (Young, Thomson et al. 2002).

1.3.1.3. *Prostaglandins*

Prostaglandins are important inflammatory mediators found in virtually all tissues and organs. They are synthesized from arachidonic acid, which is released from phospholipid membrane stores by the action of phospholipases. Free arachidonic acid is then converted to the prostaglandin intermediate prostaglandin H_2 (PGH_2) via another intermediate prostaglandin G_2 (PGG_2) by the action of prostaglandin-endoperoxidase synthase (PTGS) 1 or 2 or both (also known as cyclo-oxygenase [COX] 1 and 2). PTGS1 is considered to be a constitutively expressed enzyme, whereas PTGS2 is inducible by cytokines, growth factors or tumour promoters (Olson 2003).

PGH_2 is rapidly converted to the prostaglandins by the action of specific prostaglandin synthases to create thromboxane, prostacyclin, prostaglandin D, prostaglandin E or prostaglandin F. Biological actions of prostaglandins are mediated by specific receptors, many of which have numerous alternative splice variants. They have multiple roles in the inflammatory response including blood flow regulation, chemotaxis, and regulation of platelet function and stimulation of pain pathways. Prostaglandins have a short half-life, and are broken down to biologically inactive metabolites by the action of 15-prostaglandin dehydrogenase (15-PGDH).

Prostaglandins have vital roles in pregnancy maintenance and the process of parturition (Gibb 1998; Challis, Sloboda et al. 2002). Their production in pregnancy is tightly regulated in the placenta, fetal membranes, myometrium and cervix throughout pregnancy via the expression of enzymes involved in their synthesis and breakdown. Tissue expression of prostaglandin receptors is also modulated.

1.3.1.4. *Matrix Metalloproteinases*

Matrix metalloproteinases (MMPs) are a large family of zinc dependent endopeptidases that have similar structure and functions, but are the product of different genes (Massova, Kotra et al. 1998). They are capable of degradation of matrix and non-matrix proteins, and are involved in a large variety of physiological processes (Nagase and Woessner 1999; Sternlicht and Werb 2001). As well as modulating inflammation through regulation of the structure and integrity of the extracellular matrix, they exert effects via cleavage of cell surface receptors, activation and inactivation of chemokines, and release of pro-apoptotic mediators.

MMPs are widely distributed through most tissues, and production may be either constitutive and/or inducible by other mediators. Many are secreted immediately after being synthesized; however inflammatory cells also store MMPs as granule products for release on activation. Tissue inhibitors of metalloproteinases (TIMPs) are the major cellular inhibitors of MMPs, and antagonize their function as well as regulating their release and activation (Baker, Edwards et al. 2002).

MMPs are crucial in a number of physiological processes in the female reproductive tract, including tissue remodelling associated with ovulation and menstruation. In pregnancy, regulation of MMPs and TIMPs is vital for successful embryo implantation, trophoblast invasion and decidualization, membrane degradation at parturition and post-partum uterine involution (Fata, Ho et al. 2000).

1.3.1.5. *Toll-like receptors*

In addition to being driven by cytokines such as IL-1, TNFα and IL-17, inflammation can be directly stimulated by microbes. These provoke the innate immune response through pattern-recognition receptors such as Toll-like receptors (TLR). TLRs are members of the IL-1 receptor superfamily, and they share a cytoplasmic domain with IL-1R termed the Toll-like/interleukin receptor (TIR) domain (O'Neill 2000).

Microbial recognition by TLRs occurs via conserved pathogen motifs called pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). PAMPs are specific to micro-organisms and usually essential for the survival or immunogenicity of the pathogen. Examples include lipopolysaccharide (LPS) of Gram negative bacterial cell walls, lipotechioic acid (LTA) or peptidoglycan of Gram-positive bacterial cell walls, zymosan of yeasts, and double stranded (ds) RNA of viruses.

Toll-like receptors (TLRs) are a major group of receptors which recognise PAMPs. To date 11 TLRs have been identified (TLR 1-11) in the human (Miggin and O'Neill 2006). TLR4 was the first TLR to be discovered in humans (Medzhitov, Preston-Hurlburt et al. 1997), and its recognition of LPS is well described (Palsson-McDermott and O'Neill 2004). The ancillary proteins LPS-binding protein (LBP), CD14 and MD-2 are required for optimal TLR4 signalling. LPS is transported in serum by LBP which transfers LPS to CD14 at the cell surface. CD14, which may be membrane bound or soluble, exhibits high affinity binding for LBP bound LPS, but itself lacks an intracellular signalling domain. It is crucial for presentation of LPS to the TLR4 signalling complex. MD-2 is a small secreted glycoprotein associated with the extracellular region of TLR4, and is essential for LPS binding.

Other TLRs recognize different molecular signatures of a pathogen class (Medzhitov and Janeway 2000). TLR1 and 6 act in conjunction with TLR2, which recognizes peptidoglycan, LPS and zymosan, whereas TLR5 is stimulated by flagellin, a constituent of bacterial flagella. TLR3, 7, 8 and 9 are expressed intracellularly on the endosome membrane, and recognize intracellular pathogens. TLR3 reacts to viral dsRNA, whereas TLR7 and 8 is stimulated by viral single-stranded (ss)RNA and TLR9 is essential in CpG DNA recognition (Takeda and Akira 2005).

In pregnancy, TLR 2 and 4 have been identified in placental tissue (Holmlund, Cebers et al. 2002; Abrahams, Bole-Aldo et al. 2004; Kumazaki, Nakayama et al. 2004; Kim, Romero et al. 2005; Rindsjo, Holmlund et al. 2007) and fetal membranes (Kim, Romero et al. 2004). There is conflicting evidence regarding lower genital tract TLR expression in the human. One study found TLR 1, 2, 3, 5 and 6, but not TLR4 in the vagina and ectocervix (Fazeli, Bruce et al. 2005) whereas another has identified TLR4 in the vagina (Pivarcsi, Nagy et al. 2005). TLR 1-9 mRNA has also been identified in the vagina and uterine tissues of the mouse (Soboll, Schaefer et al. 2006). TLR 1, 2, 3, 4, 5 and 6 are epithelially expressed in the human endocervix, endometrium and fallopian tubes in the non-pregnant female reproductive tract (Fazeli, Bruce et al. 2005). TLR2 mRNA and protein expression is higher in the fallopian tube and cervix than in the endometrium and ectocervix, whereas TLR4 expression is greater in the fallopian tube and endometrium (Pioli, Amiel et al. 2004). Moreover, it has recently been demonstrated that TLR 2, 3, 4 and 9 mRNA expression in the endometrium alters during the menstrual cycle (Hirata, Osuga et al. 2007).

1.3.2. Inflammatory Signalling

Inflammatory gene expression is usually dependent on transcriptional activation, and the gene promoters of many inflammatory mediators contain binding sites for the nuclear factor kappa-B (NFκB), activated protein-1 (AP-1), CAAT/enhancer-binding protein (C/EBP) and/or erythroblast transformation specific domain transcriptional regulators (ETS). Mutation or deletion of these elements has been shown to impair

the induction of promoters of chemokines, PGTS2, adhesion molecules and matrix metalloproteinases essential in the inflammatory response (Kracht and Saklatvala 2002). Signals from TLR/ILRs, and the distinct TNFα receptors and IL-17 receptors, all converge on pathways which are pivotal in regulation of these transcription factors – the NFκB and mitogen-activated protein kinase (MAPK) pathways. Multiple levels of regulation occur in these pathways, as outlined below. This enables a finite number of stimuli to initiate distinct, but related responses, depending on the physiological and cellular conditions.

A diagrammatic overview of inflammatory signalling is given in Figure 1.2.

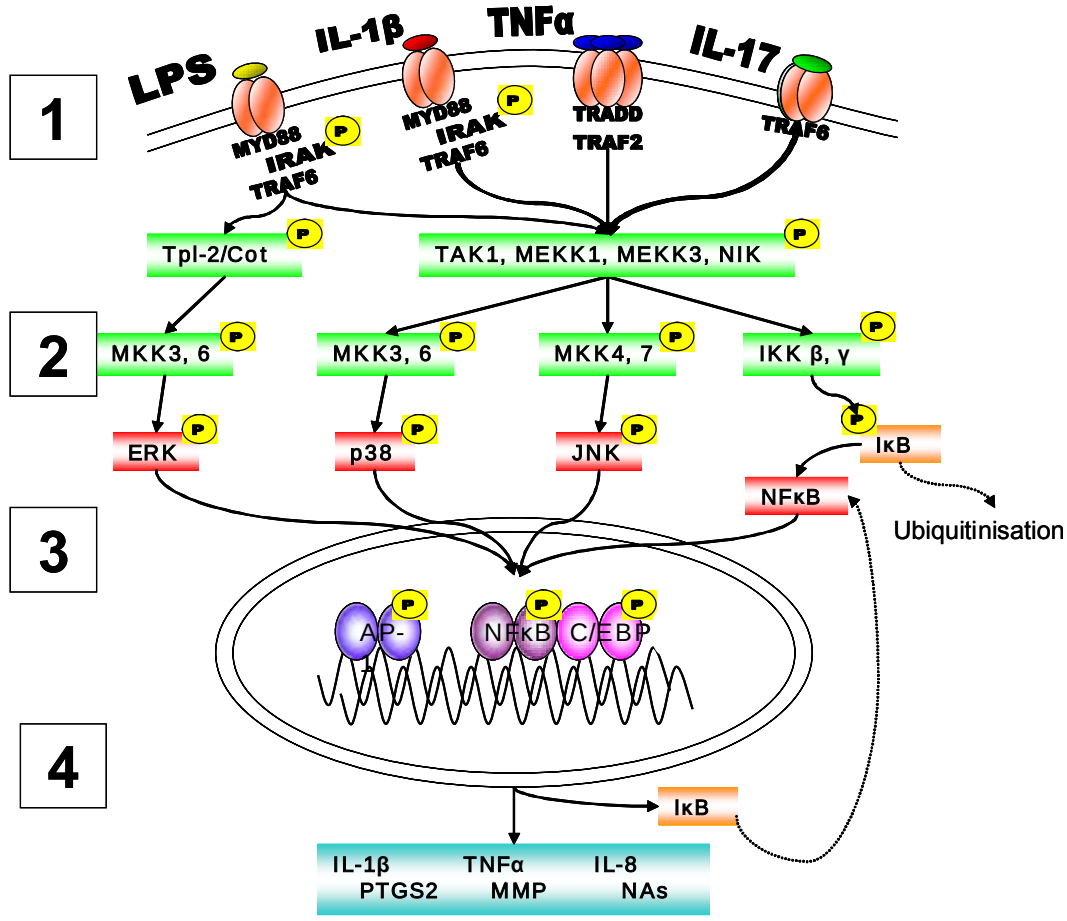


Figure 1.2

Overview of Inflammatory Signalling (adapted from Kracht and Saklatvala 2002).

1. Receptor activation and recruitment of adaptor proteins
2. Protein Kinase Cascade
3. Transcription factor nuclear localization and transcription
4. mRNA stabilization and post-transcriptional modifications

(See text for details.)

1.3.2.1. *Receptor activation and adaptor recruitment*

On ligand activation IL-1R heterodimerizes with an accessory protein (IL-1R-AcP) whilst TLRs oligomerize and TNFRs trimerize. These complexes interact with cytosolic adaptor proteins which are essential for further signal transmission. TNFR binds to the adaptor TRADD (TNF-receptor-death domain), whilst TIRs bind to an adaptor called MYD88 (myeloid differentiation primary response gene 88). These in turn, recruit other adaptors – TRAFs (TNF Receptor-associated factors). TRADD directly recruits TRAF-2, whereas MYD88 engages TRAF-6, via phosphorylation of an IL-1R-associated kinase (IRAK). TRAF-6 also binds directly to liganded IL-17R (Kracht and Saklatvala 2002).

Additional adaptors have recently been discovered which add to the complexity of the process. MAL (MYD88 Activator Like Protein), TRIF (TIR domain containing adaptor inducing interferon-beta) and TRAM (TRIF-related activator molecule) interact with defined TLRs, and four IRAKS and several members of the TRAF family have now been identified. Regulation of signalling can occur though sequestration, phosphorylation and degradation of these adaptor proteins, and interaction with negative regulatory adaptors such as IRAK-M or Toll-interacting protein (TOLLIP) (Miggin and O'Neill 2006).

1.3.2.2. *Activation of protein kinase cascades*

Downstream stimulation of inflammatory signalling pathways occurs via activation of IκB kinases (IKKs) in the NFκB pathway, and MAPK-kinases in the MAPK pathway. A large number of molecules have been implicated in the activation of these proteins, depending on the stimulus and cellular conditions (for reviews see (Kyriakis and Avruch 2001; Hayden and Ghosh 2004; Scheidereit 2006), but compensatory mechanisms and functional redundancy make elucidation of their roles difficult. Several enzymes can regulate kinases of both the NFκB and MAPK pathways (eg Mitogen activated/Erk kinase kinase or MEKK1, 2 and 3; MAP3K8; and TGFβ activated kinase-1 or TAK-1) and may activate two or more cascades simultaneously. Substantial cross-talk between the pathways is recognized.

IκB kinases (IKKs) and the NFκB pathway

Inhibitors of κB (IκBs) are proteins which maintain NFκB dimers in an inactivated form in the cellular cytosol. Inflammatory stimuli induce IκB degradation via IKKs. IKKα, IKKβ and the scaffold protein IKKγ (also known as NEMO; NFκB essential modulator) form a complex, which represents a point of convergence of signals that activate NFκB by the so-called canonical pathway (Karin and Delhase 2000). IKKβ and NEMO are essential for IκB phosphorylation, which then rapidly undergoes polyubiquitinisation and is degraded via the 26s proteosome. This releases NFκB transcription factor subunits allowing them to translocate to the nucleus.

A slower non-canonical pathway of NFκB activation, which is dependent on IKKα, is also recognized. IKKα is phosphorylated by NFκB inducing kinase (NIK), which is activated by a subset of inflammatory stimuli including lymphotoxin β and the CD40 ligand, although they can also use the canonical pathway (Bonizzi and Karin 2004). Other IKK independent mechanisms also exist, which have not yet been fully elucidated (Gilmore 2006).

MAPK-kinases and the MAPK pathway

MAPKs (ERK, JNK and p38) are terminal enzymes in related signalling cascades. There are two forms of ERK (ERK1 and 2), three JNKs (JNK 1, 2 and 3) and three forms of p38 (p38α, β and γ). Each is activated by dual phosphorylation by a MAPK-kinase (MKK). MKK1 and 2 activate ERK 1 and 2; MKK4 and 7 synergistically activate JNK; and MKK 3 and 6 activate p38. Although the MAPK pathways are distinct, there is some overlap in MAPK substrate specificity; for example all three MAPKs activate the ETS transcription factors ELK1 and SAP (senescence-associated protein) 1 and 2 (Kyriakis and Avruch 2001). Negative regulation of transcription factors phosphorylated by MAPKs can occur via interactions with scaffold and adaptor proteins which retain them in the cytosol (Kracht and Saklatvala 2002).

Inflammatory and stressful stimuli nearly always activate JNK and p38, but their effect on ERK is more variable (Kyriakis and Avruch 2001). ERK can also be

stimulated by mitogens and hormones and many of the putative substrates for ERK are involved in general cellular processes.

1.3.2.3. *Transcription factors*

The NFκB and MAPK pathways can activate members of a variety of transcription factor families. There is substantial cross-talk between the pathways.

AP-1

AP-1 consists of homodimers and/or heterodimers of basic region leucine zipper proteins of the c-Jun and c-Fos and the related activating transcription factor (ATF) subfamilies (Karin, Liu et al. 1997). Regulation of AP-1 activity occurs via phosphorylation of existing subunits, as well as novel gene expression. Dimer composition is influenced by the extracellular stimulus and the signalling cascade which is activated, and the differential makeup of AP-1 complexes influences their DNA binding capacity and capability to mediate gene expression.

NFκB

NFκB is a family of dimeric proteins which bind to DNA κB elements. These consist of 9–10 base pairs but exhibit a great amount of variability. The group is comprised of two subfamilies; the NFκB proteins (in the human NFκB1 and NFκB2; also known as p105 and p100) and the Rel proteins (in the human Rel A also known as p65; Rel B and C-Rel) (Gilmore 2006). They all share a highly similar domain of around 300 amino acids known. This is known as the Rel homology domain (RHD) and contains the motif for binding κB elements, as well as a motif for dimerization, and a motif for nuclear localization (the nuclear localization signal or NLS) (Baldwin 1996).

NFκB1 and 2 (p100 and p105) are precursors which are converted to active DNA binding forms, respectively known as p50 and p52, by proteolysis or arrested translation (Gilmore 2006). They have long carboxy-terminal domains containing multiple copies of ankyrin repeats which inhibit their transcription ability. As such, NFκB protein homodimers may act as inhibitors of gene expression in resting cells,

and generally only activate transcription when they dimerize with Rel proteins, which do possess transactivation domains.

In vivo all NFκB subunits can form homodimers, except for Rel B, which can only form heterodimers. Individual dimers have distinct DNA-binding site specificities, and form different protein-protein interactions at target promoters, thus allowing regulation of distinct, but overlapping genes, many of which are involved in inflammation.

C/EBP

C/EBP is a family of six basic region leucine zipper protein transcription factors, which contribute to the expression of a number of inflammatory mediators including chemokines (Stein and Baldwin 1993) and PGTS2 (Caivano, Gorgoni et al. 2001). They bind in different combinations to promoters, replacing or synergizing with other factors to maintain gene activation (Poli 1998) and their activity is at least partially dependent on phosphorylation by a number of protein kinases.

ETS domain transcription factors

ETS-domain transcription factors are a large family of proteins that bind to an ETS DNA-binding element (Yordy and Muise-Helmericks 2000; Sharrocks 2001). Several are important in the regulation of inflammatory genes, including ETS1, ELK1 and SAP1. They mediate expression either by direct binding to a promoter, or via binding to serum-response elements (SRE) which are found on the promoters of some inflammatory genes. The promoters of other transcription factors such as c-Fos and Jun B also contain SREs, thus ETS domain transcription factors can indirectly induce expression of genes regulated by these molecules.

1.3.2.4. *Inflammatory gene repression*

Several mechanisms repress inflammatory gene expression in unstimulated cells. Examples are the deacetylation of histones on gene promoters; DNA binding of inhibitory units such as OCT (octamer binding protein)1; and the actions of repressor proteins for example the NFκB repressor NRF (Kracht and Saklatvala 2002). Inflammatory stimuli may enable transcription through removal of these brakes ie by

the acetylation of histones and promoter remodelling; the displacement of inhibitory units by NFκB or C/EBP; and the conversion of co-factors with repressing functions to ones with stimulatory functions. Such processes may have evolved to prevent uncontrolled activation of the inflammatory system.

1.3.2.5. *mRNA stabilization and post-transcriptional modifications*

Many pro-inflammatory genes are inherently unstable, and may be stabilized by components of the MAPK or NFκB pathways (Ross 1995). This provides another level of control in the inflammatory response, allowing rapid changes in levels of gene expression and protein production to be promptly terminated. Post-transcriptional modifications can also occur, further regulating protein expression.

1.4. INFLAMMATION, INFECTION AND PRETERM LABOUR

1.4.1. Labour as an inflammatory process

There is a substantial body of evidence suggesting that normal labour is an inflammatory process. Levels of IL-1β (Romero, Parvizi et al. 1990; Gunn, Hardiman et al. 1996; Laham, Brennecke et al. 1996), TNFα (Romero, Mazor et al. 1992; Laham, Brennecke et al. 1994) and IL-8 (Laham, Rice et al. 1993; Saito, Kasahara et al. 1993) rise in the amniotic fluid in association with normal labour. Findings in animal models suggest that this is a cause of the process, rather than a consequence of parturition, as intra-amniotic infusion of IL-1β and TNFα can stimulate labour (Romero, Mazor et al. 1991; Sadowsky, Adams et al. 2006), and IL-1β, TNFα and IL-8 can stimulate cervical ripening (Chwalisz, Benson et al. 1994). Leukocytes which infiltrate the myometrium, cervix and fetal membranes at or around the onset of labour (Young, Thomson et al. 2002; Osman, Young et al. 2003) are a source of inflammatory cytokines and chemokines. In addition, the fetal membranes, decidua and placenta may contribute to cytokine production (see Table1.1).

Prostaglandins are crucial in the process of parturition. These can stimulate myometrial contractions directly (Crankshaw and Dyal 1994) and indirectly via

upregulation of oxytocin receptors and synchronization of contractions (Liggins 1989). They can also stimulate cervical ripening (Kelly 2002) and are implicated in fetal membrane rupture via induction of matrix metalloproteinases (McLaren, Taylor et al. 2000; Ulug, Goldman et al. 2001). Levels of prostaglandins rise in the maternal serum and fetal membranes before or at the time of labour (Olson 2003). This rise appears to be mediated, at least in part, by upregulation of PTGS2 in the decidua (Hirst, Mijovic et al. 1998; Mijovic, Zakar et al. 1999), fetal membranes (Mijovic, Zakar et al. 1997; Slater, Dennes et al. 1999; Sawdy, Slater et al. 2000) and myometrium (Sooranna, Grigsby et al. 2006). PTGS2 inhibitors decrease uterine contractility in vitro (Sadovsky, Nelson et al. 2000; Sawdy, Pan et al. 2003) and delay birth and prolong pregnancy *in vivo* (Panter, Hannah et al. 1999).

Changes in NFκB activity are evident within the fetal membranes (Allport, Pieber et al. 2001), myometrium (Chapman, Europe-Finner et al. 2004) and cervix (Stjernholm-Vladic, Stygar et al. 2004) at the time of labour. Furthermore, NFκB, AP-1 and C/EBP regulate expression of PGTS2 (Allport, Pieber et al. 2001; Mohan, Sooranna et al. 2007), cytokines (Lappas, Permezel et al. 2002), matrix metalloproteinases (Lappas, Permezel et al. 2003) and oxytocin receptors (Terzidou, Lee et al. 2006) in the uterine tissues.

1.4.2. The onset of normal labour

Although it is recognized that labour is associated with an inflammatory response, the way this process is normally initiated in the human remains poorly understood. In the sheep the onset of uterine activity is mediated by a combination of progesterone withdrawal and increased prostaglandin production, caused by increased adrenocortical activity in the fetus at the end of gestation. This leads to high levels of circulating PGE_2 and cortisol which shifts placental steroid synthesis to favour oestrogen over progesterone. There is also an increase in placental PTGS2 activity in response to fetal corticotrophin releasing hormone (CRH) (Challis, Matthews et al. 2000).

There is no apparent progesterone withdrawal at the end of human pregnancy, and circulating levels of progesterone remain high until birth. Nevertheless, many of the genes which are upregulated at the time of labour are repressed by progesterone, and antiprogesterones can cause cervical ripening and induce the termination of pregnancy (Kelly 1994). It has thus been suggested that a functional progesterone withdrawal coincides with the onset of human labour (Brown, Leite et al. 2004). This may occur via the catabolism of progesterone within the uterus to inactive compounds by enzymes such as progesterone 5 alpha-reductase. Mice deficient in the type 1 isozyme of this enzyme fail to deliver their young at term despite initiation of co-ordinated myometrial contractions. This parturition defect appears to correspond to accumulation of progesterone in the cervix and failure of cervical ripening (Mahendroo, Porter et al. 1999). Functional progesterone withdrawal could also be mediated by alterations in the ratio of progesterone receptor isoforms (Thijssen 2005), or changes in cofactor levels which affect receptor transactivation (Condon, Jeyasuria et al. 2003). An alternative mechanism may be through the activation of NFκB, which has a mutually negative interaction with the progesterone receptor (Kalkhoven, Wissink et al. 1996; Pieber, Allport et al. 2001; Condon, Hardy et al. 2006) and thus may remove the repressive effect of progesterone from labour-associated genes.

In the human, serum levels of CRH early in the second trimester correlate with gestation at delivery (McLean, Bisits et al. 1995). CRH expression in the placenta and fetal membranes increase towards time of delivery (Riley, Walton et al. 1991) and it has also been shown that *in vitro* prostaglandin synthesis by human fetal membranes can be up-regulated by CRH (Alvi, Brown et al. 1999). However no direct link between CRH and onset of labour has been proven.

1.4.3. The onset of preterm labour

Normal labour occurs between 37 and 42 weeks gestation, whereas preterm labour is that occurring before 37 completed weeks. Multiple aetiologies contribute to the onset of preterm labour, through a variety of pathological processes. These include uterine distension, cervical disease, ischaemia, endocrine disorders, allergic

phenomena, abnormal allograft reaction and infection (Romero, Espinoza et al. 2006).

Although the initiating stimuli vary, they all usually lead to delivery through the common pathway of cervical dilatation, myometrial contractions and membrane rupture seen in normal labour. It is hypothesized that these insults all, in some way, instigate a vigorous inflammatory response, which overwhelms normal inhibitory mechanisms and results in irreversible stimulation of the parturition cascade. Consistent with this theory, is the observation that levels of inflammatory mediators in gestational tissue exposed to preterm labour are higher than those seen in association with normal labour (Bowen, Chamley et al. 2002; Keelan, Blumenstein et al. 2003).

1.4.4. Infection as a cause of preterm labour

A particularly exaggerated inflammatory response is seen in the fetal membranes and amniotic fluid in cases of infection-associated preterm labour (Bowen, Chamley et al. 2002; Keelan, Blumenstein et al. 2003). Evidence that infection is causative of both the inflammation and the labour is provided by animal studies. These have proven that administration of bacteria or bacterial products stimulates labour; that resultant increases in inflammatory mediator levels precede the onset of uterine contractions; and that adjacent antibiotic therapy can delay or prevent the process (Table 1.2).

In obstetric practice, an association between extrauterine infections such as pyelonephritis and pneumonia, and preterm labour has long been recognized. In addition, clinical chorioamnionitis, which is characterized by features of maternal pyrexia, uterine tenderness and fetal tachycardia is frequently an antecedent of preterm delivery. There is now a substantial body of evidence suggesting subclinical bacterial infections are also a cause of preterm delivery. This is provided by studies involving the microbiological analysis of amniotic fluid samples. Much of the data comes from the USA, where amniocentesis is frequently performed on women presenting with threatened preterm labour for the diagnosis of infection. This

practice is unusual in the UK, so there is a lack of corresponding data from this country.

	Agent	Preterm Delivery Rate	Delivery Interval	Reference and Notes
RAT				
Systemic	LPS	100%	Approx 25 h	(Celik and Ayar 2002) *Erythromycin prolongs latency period*
Intrauterine	LPS	Unclear	82±13 and 63±8 h, respectively (controls, 117±3 h)	(Bennett, Terrone et al. 2000) *Uterine catheters implanted on day 16; LPS infused on day 17 (term day 22)*
MOUSE				
Systemic	LPS	Variable; 100% with C3H/HEN dams	Not reported	(Kaga, Katsuki et al. 1996) *Preterm delivery rate depended on mated strains*
	LPS	90%	Not reported	(Lee, Kim et al. 2003) *20% of mice receiving i.p. saline deliver preterm*
	LPS	100%	Within 24 h	(Gross, Imamura et al. 2000) *PTGS2 inhibitors decrease preterm birth but do not preserve fetal viability*
	IL-1	100%	Within 24 h	(Romero, Mazor et al. 1991) *Study done in C3H/HEJ (Tlr4 mutant) mice*
	LPS	70%	Not reported	(Harper and Skarnes 1973) *Study done in CD-1 mice; treatment with progesterone decreases preterm birth but results in no fetal viability*
	LPS	100%	16.8 h (95% CI 15.9–17.6) versus 54.7 h (95% CI 43.8–65.5) for saline-injected mice	(Buhimschi, Buhimschi et al. 2003) *N-acetylcysteine increases latency interval to preterm birth in LPS-treated mice*
	Fuso-bacterium nucleatum	Difficult to assess (term stillbirths included with preterm delivery in analysis)	Not reported	(Han, Redline et al. 2004) *Study done in CF-1 mice; F bacterium dental pathogen; causes preterm delivery and fetal demise*
	LTA	100% at 75 mg/kg	Not reported	(Kajikawa, Kaga et al. 1998) *C3H/HEN mated with B6D2F1 mice; higher dose of LTA than LPS required to induce preterm delivery*
Intrauterine	LPS	91%	Within 20 h	(Elovitz, Wang et al. 2003) *No maternal mortality*
	Escher-ichia coli	100% at 10^{10} CFU	Not reported	(Mussalli, Blanchard et al. 1999) *10^{7} CFU or less does not induce preterm delivery; two maternal deaths with higher inoculums*
Intracervical	*Escher-ichia coli*	100%	Within 36 h	(Reznikov, Fantuzzi et al. 1999) *Il-1β-/-$^{-}$ mice have same rates of preterm delivery; TNF antagonist does not prevent preterm delivery*
RABBIT				
Intrauterine	LPS	Induced or augmented contractions	Preterm delivery not an outcome	(Katsuki, Kaga et al. 1997) *LPS infused through intrauterine catheters*
Intracervical	*Escher-ichia coli*	100%	within 1–2 days (n=5)	(Davies, Shikes et al. 2000) *Introduced into cervix via hysteroscopy*
	Prevotella bivia	33% (3/9) have preterm delivery without maternal fever	Not reported	(Gibbs, McDuffie et al. 2004) *P. bivia associated with bacterial vaginosis*

RHESUS MONKEY				
Chorio decidual space	Group B Strepto-coccus	Preterm contractions; cervical ripening several days later	Not reported	(Gravett, Haluska et al. 1996) *Increased MMP-9 levels in amniotic fluid before GBS detected in amniotic fluid*
Intra-amniotic	Group B Strepto-coccus	100% (*n*=4) show increased intra-amniotic cytokines followed by regular contractions	Not reported	(Gravett, Witkin et al. 1994) *No monkeys are febrile or have leukocytosis at the time of preterm delivery*
	IL-1	100% (n=5)	1.1+/-0.2 days (27.6=/-1.9 in saline infused controls)	(Sadowsky, Adams et al. 2006) *Elevation in amniotic Il-1β, Il-6, Il-8, prostaglandins, MMP9 and leikocytes*
	TNFα	40% delivered preterm; 100% (n=5) showed increased contractions	Not analyzed	(Sadowsky, Adams et al. 2006) *Elevation in amnioticIL-1β, IL-6, Il-8, prostaglandins, MMP9 and leukocytes*
	IL-8	0% (n=2)	16.1-23 days	(Sadowsky, Adams et al. 2006)
	IL-6	0% (n=2)	23.7-29.7days	(Sadowsky, Adams et al. 2006)
SHEEP				
Systemic	Salmonella LPS	Either 4–5 ewes delivered preterm or have fetal death	Within 28 h	(Schlafer, Yuh et al. 1994) *All fetuses become hypoxic; maternal and fetal cortisol increases*
Intra-amniotic	*Escher-ichia coli* LPS	0% (n=4)	None delivered preterm	(Grigsby, Hirst et al. 2003) *Small but significant increase in contractile activity noted in all sheep*
Extrauterine	Constant infusion of LPS	0%	None delivered preterm	(Grigsby, Hirst et al. 2003) *No observable effect on uterine contractile activity*

Table 1.2

Animal models used to study infection/inflammatory preterm labour (adapted from (Elovitz and Mrinalini 2004)).

The amniotic cavity is normally sterile. Women with positive amniotic cultures are more likely to have preterm delivery, spontaneously rupture their membranes, develop clinical chorioamnionitis and/or have an adverse perinatal outcome (Romero, Espinoza et al. 2002). Positive culture rates of 12.8% are found in women presenting with threatened preterm labour with intact fetal membranes, and in 22% of those who actually go on to deliver prematurely (Goncalves, Chaiworapongsa et al. 2002). Preterm prelabour rupture of membranes (PPROM) commonly precedes preterm delivery, and PPROM occurring in early gestation is that which is most likely to be associated with positive amniotic fluid cultures (Watts, Krohn et al. 1992). 32.4% of all women with PPROM have positive amniotic fluid cultures on admission, which rises to 75% at the time of onset of labour, suggesting that intrauterine microbial invasion can occur in the interim between membrane rupture and the start of contractions (Goncalves, Chaiworapongsa et al. 2002). Studies which have identified the presence of bacteria in the amniotic fluid by PCR methods, which are more sensitive than culture techniques, have shown even higher incidences of intra-amniotic infection (Jalava, Mantymaa et al. 1996; Hitti, Riley et al. 1997; Gardella, Riley et al. 2004), with similar rates of adverse outcomes (Yoon, Romero et al. 2003).

The most common way for bacteria to access the uterine cavity is by ascension from the vagina and cervix. Haematogenous spread of bacteria through the placenta may also occur. This is the putative mechanism explaining the recent finding of *Fusobacterium nucleatum*, an oral pathogen, in the amniotic fluid of women with preterm labour (Bearfield, Davenport et al. 2002). An association between preterm labour and periodontal disease is recognized but a direct link between the two conditions has not been proven (Klebanoff and Searle 2006). Rarer methods of bacterial spread are from the peritoneal cavity by way of the fallopian tubes, and iatrogenic infection from instrumentation of the amniotic cavity.

Once bacteria reach the uterine cavity they can elicit an inflammatory reaction in the decidua and, if not contained, the contiguous fetal membranes. Bacteria are frequently detected in the chorioamnion in cases of preterm labour. However,

fluorescent in *situ* hybridization (FISH) can detect bacteria in the fetal membranes of up to 70% of women undergoing prelabour Caesarean section at term, implying that colonisation of the chorioamnion is not sufficient for the stimulation of labour or penetration of the amniotic cavity (Steel, Malatos et al. 2005).

It is likely that environmental factors and pathogen virulence contribute to the establishment of chorioamnionitis. Genetic variation in the inflammatory response could also be an important factor and may explain the marked racial differences in the incidence of preterm labour which are seen. Single nucleotide polymorphisms in the TLR4 gene are more frequent in infants born at preterm gestations (Lorenz, Hallman et al. 2002). Polymorphisms in genes coding for IL-1β (Genc, Gerber et al. 2002) and TNFα (Simhan, Krohn et al. 2003) are seen in increased frequency in association with preterm labour in a black population, whilst a polymorphism of the IL-6 gene resulting in decreased expression may be protective against preterm labour (Simhan, Krohn et al. 2003). Multilocus analysis of several allelles may reveal stronger genetic associations with premature birth (Menon, Velez et al. 2006).

Retrospective analysis of amniotic fluid obtained for genetic diagnosis in the second trimester of pregnancy has shown that the presence of bacteria (Cassell, Davis et al. 1983; Gray, Robinson et al. 1992; Horowitz, Mazor et al. 1995) and raised levels of IL-6 (Wenstrom, Andrews et al. 1996; Ghidini, Jenkins et al. 1997) and MMP-8 (Yoon, Oh et al. 2001; Biggio, Ramsey et al. 2005) correlate with preterm delivery and pregnancy loss in the third trimester. This implies that infection can occur silently in pregnancy, stimulating an inflammatory response which leads to preterm loss or delivery only some weeks later. The organisms most commonly found associated with preterm labour are low virulence bacteria including *Mycoplasma hominis, Ureaplasma urealyticum, Gardnerella vaginalis, Peptostreptococci spp* and *Bacteroides* (Romero, Sirtori et al. 1989). Long term, low grade inflammatory stimulation by such microbes may thus be important in the pathogenesis of preterm labour.

1.4.5. Bacterial vaginosis and preterm labour

Bacterial vaginosis is a polymicrobial infection of the lower genital tract characterized by a deficit of the normal population of lactobacillus, and overgrowth of low virulence mixed anaerobic bacteria including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Bacteroides* spp, *Peptostreptococcus* spp, *Fusobacterium* spp, *Prevotella* spp, *Mobiluncus* spp (Lamont, 2004). Sufferers may complain of a malodorous vaginal discharge, but it rarely causes vulval itch, is not associated with a classical inflammatory reaction and in the majority of cases is asymptomatic.

There is substantial evidence showing a relationship between bacterial vaginosis and poor perinatal outcome, in particular preterm labour, rupture of membranes and late miscarriage (McGregor, French et al. 1990; Kurki, Sivonen et al. 1992; Hay, Lamont et al. 1994; Hillier, Nugent et al. 1995) . Women with bacterial vaginosis are also at increased risk of a number of other infectious pathologies, such as pelvic inflammatory disease, HIV (Human immunodeficiency virus) infection, post-operative infections and post-partum endometritis (Boyle, Adinkra et al. 2003).

A meta-analysis of case-control and cohort studies showed bacterial vaginosis increases the risk of delivery before 37 weeks with an odds ratio of 2.19 (CI 1.54-3.12) (Leitich, Bodner-Adler et al. 2003). The rates of preterm labour were much higher in subgroup analyses where bacterial vaginosis was diagnosed before 20 weeks and 16 weeks gestation, however other studies have not shown this association (Klebanoff, Hillier et al. 2005). Trials investigating the effect of eradication of bacterial vaginosis on pregnancy outcome have given contradictory results, and it is unclear as to whether this increased risk of preterm labour is a direct result of bacterial vaginosis, or if bacterial vaginosis is a surrogate marker of an alternative underlying pathology. A systematic review of the treatment of bacterial vaginosis in pregnancy has shown little evidence that eradication of bacterial vaginosis results in any reduction in the incidence of preterm delivery in a low risk population, although some benefit may be seen with treatment before 20 weeks gestation (McDonald, Brocklehurst et al. 2007). Treatment of women who are at high risk of preterm

labour (who have had a previous preterm delivery) conveys slight protection over preterm rupture PPROM and low birth weight, but not preterm delivery (McDonald, Brocklehurst et al. 2007). Common treatment regimes for bacterial vaginosis include metronidazole and clindamycin given orally or vaginally. Although both are effective at eradicating bacterial vaginosis, this review found no benefit of clindamycin or intravaginal metronidazole in relation to pregnancy outcome.

Two methods are commonly used for the diagnosis of bacterial vaginosis in research studies. Amsel's composite criteria provide a clinical diagnosis made by finding three of the following four signs: a homogenous vaginal discharge; an elevated vaginal pH (>4.5); clue cells on wet preparation of vaginal secretions; a "positive whiff test" (amine odour) on addition of a 10% solution of potassium hydroxide to a sample of vaginal secretions (Amsel, Totten et al. 1983). However, these criteria are subjective, poorly reproducible and unpleasant. Analysis of a Gram stain of air dried vaginal secretions using Nugent's criteria (Nugent, Krohn et al. 1991) provides a method of diagnosing bacterial vaginosis which is objective and reproducible, as well as being cheap, quick and simple. It has therefore largely replaced Amsel's criteria as being the gold standard for diagnosis. The technique has the advantage of identifying a third group of vaginal flora, intermediate in character, which is abnormal, but less florid than that found in bacterial vaginosis (Hillier, Krohn et al. 1992; Rosenstein, Morgan et al. 1996; Taylor-Robinson, Morgan et al. 2003). Diagnosis is made by quantifying the normal and abnormal vaginal flora to obtain a score between 1 and 10, with 0-3 being normal (Grade I), 4-6 representing intermediate bacterial vaginosis status (Grade II), and 7-10 representing bacterial vaginosis (Grade III). In clinical practice techniques for diagnosis of bacterial vaginosis are still variable, and heterogeneity in the method used results in differences in perceived prevalence of the condition and treatment of it (Keane, Maw et al. 2005).

Bacterial vaginosis is generally thought of as a non-inflammatory condition, and the number of vaginal polymorphonuclear cells found in bacterial vaginosis is not increased with respect to that in healthy women (Cauci 2004; Ramsey, Lyon et al.

2005). Nevertheless levels of inflammatory cytokines are increased in vaginal secretions when the condition is present. Vaginal IL-1β concentrations are higher in women with bacterial vaginosis than in those with normal flora, in the pregnant (Imseis, Greig et al. 1997; Mattsby-Baltzer, Platz-Christensen et al. 1998; Cauci, Guaschino et al. 2003) and non-pregnant (Spandorfer, Neuer et al. 2001; Hedges, Barrientes et al. 2006) states; and return to normal after successful treatment of the condition (Basso, Gimenez et al. 2005). IL-1β levels are also higher in women with intermediate flora (Hedges, Barrientes et al. 2006). IL-8 concentrations have been found to be elevated in vaginal secretions from women with bacterial vaginosis in some studies (Wennerholm, Holm et al. 1998; Spandorfer, Neuer et al. 2001; Basso, Gimenez et al. 2005), but these findings are not confirmed in other groups' work (Cauci, Guaschino et al. 2003; Diaz-Cueto, Cuica-Flores et al. 2005). There are also mixed reports about levels of IL-1α (Platz-Christensen, Mattsby-Baltzer et al. 1993; Imseis, Greig et al. 1997; Wennerholm, Holm et al. 1998). These differences probably relate to differences in sample collection and sensitivity of analysis. Levels of IL-6 and TNFα (Imseis, Greig et al. 1997; Hedges, Barrientes et al. 2006) do not seem to be altered with bacterial vaginosis in vaginal secretions, although levels of both IL-1β and TNFα are raised in cervical mucus in the presence of bacterial vaginosis (Sturm-Ramirez, Gaye-Diallo et al. 2000). There is no evidence of concurrent elevation of serum cytokines in the presence of bacterial vaginosis (Hedges, Barrientes et al. 2006).

The prevalence of bacterial vaginosis varies with the population studied, being found in 15% of pregnant women in a middle-class area of London (Hay, Lamont et al. 1994), and 33% of pregnant women in an American inner city area (McGregor, French et al. 1995). There is considerable racial disparity, being more common in Afro-Caribbean women than Caucasian women (Goldenberg, Klebanoff et al. 1996; Royce, Jackson et al. 1999). The condition is associated with a number of factors including multiple sexual partners (Morris, Rogers et al. 2001; Smart, Singal et al. 2004), lesbian sexual activity (Bailey, Farquhar et al. 2004), cigarette smoking (Hellberg, Nilsson et al. 2000; Smart, Singal et al. 2004), intrauterine contraceptive

device usage (Joesoef, Karundeng et al. 2001) and vaginal douching (Ness, Hillier et al. 2002).

Genetic variation among individuals may be responsible for an increased susceptibility to both bacterial vaginosis and preterm delivery, and polymorphisms in genes for IL-1β, IL-6, IL-8 and TLR4 have been associated with increased rates of bacterial vaginosis in pregnancy (Genc, Vardhana et al. 2004; Goepfert, Varner et al. 2005). However, one study has found that a TNFα promoter polymorphism predisposes to preterm delivery only in the presence of bacterial vaginosis (Macones, Parry et al. 2004), suggesting gene-environment interactions are crucial to the development of preterm labour. These findings may go some way to explaining the conflicting results of bacterial vaginosis treatments on pregnancy outcome.

1.4.6. Clinical aspects of preterm labour and infection

1.4.6.1. *Outcomes*

Preterm birth is associated with high rates of perinatal mortality, as well as short and long term morbidities which have a substantial burden on healthcare resources. In Scotland in 2004, 7.8% of births occurred before 37 weeks, and these accounted for 64% of neonatal deaths (Scottish Morbidity Report 02 and Scottish Perinatal Infant and Maternal Mortality Report; ISD Scotland; http://www.isdscotland.org/) In England, Wales and Northern Ireland in 2004, 73% of neonatal deaths were related to prematurity (Perinatal Mortality Surveillance, 2004, England, Wales and Northern Ireland; Confidential Enquiry into Maternal and Child Health (CEMACH); http://www.cemach.org.uk). Approximately one-third of these deliveries are iatrogenic, with delivery indicated by conditions such as severe intrauterine growth restriction or fulminating pre-eclampsia. The rest are a result of preterm labour. Despite some advances in understanding its aetiology in the last two decades, rates of preterm labour have remained static, or even slightly increased. Incidence in the developing world is even higher. As yet there is no effective way of preventing preterm labour, predicting it remains difficult and there are only limited treatment options.

Infection is responsible for 30-50% of cases of preterm labour. When infection is associated with preterm labour, the perinatal outcome is worsened. This is probably secondary to activation of the fetal inflammatory response by infectious organisms. Fetal bacteraemia has been detected in 33% of babies with infected amniotic fluid, compared to 4% of those with negative amniotic fluid culture (Gray, Robinson et al. 1992). Neonates which have been exposed to intrauterine infection are more likely to be affected by severe morbidities including respiratory distress, intraventricular haemorrhage, periventricular leukomalacia, bronchopulmonary dysplasia and necrotizing enterocolitis, in addition to having higher rates of sepsis (Gomez, Romero et al. 1998).

Infection also significantly worsens the perinatal outcome in term parturition (Peebles and Wyatt 2002), illustrating that even when infection is not causative of the labour process, it can have deleterious effects. The open cervix, and in some case ruptured membranes, provide a direct route for microbial ascension from the vagina, and acute infection of the uterine contents.

1.4.6.2. *Antibiotics for the prevention of preterm labour*

Long standing recognition of an association between infection and preterm labour has led to trials of antibiotics for its prevention. One intervention that has been successful is the antibiotic treatment of asymptomatic bacteriuria in pregnancy which results in a 40% reduction of preterm labour or delivery of a low birth weight infant (Smaill 2001). It is unclear whether this decrease is secondary to a reduction in subsequent acute urinary tract infection, or whether it represents clearance of pathogen colonisation of the genitourinary epithelium.

A recent meta-analysis of antibiotic prophylaxis in the second trimester of pregnancy has shown that macrolides (erythromycin, azithromycin, clarithromycin and clindamycin) are associated with a moderate decrease in the incidence of preterm birth (OR 0.72, confidence interval 0.56-0.93), but metronidazole is associated with a slight increase in preterm labour (OR 1.1, confidence interval 0.95-1.29) (Morency

and Bujold, 2007). These conflicting results, along with those involving the eradication of bacterial vaginosis discussed above, reflect both the complexity of the pathogenesis of preterm labour and the heterogeneity of interventional trials.

Indiscriminate antibiotic use can be harmful via the promotion of antibiotic resistant strains. They may also have more specific detrimental effects, as illustrated by the increased incidence of necrotizing enterocolitis associated with co-amoxiclav use in pregnancy (Kenyon, Taylor et al. 2001). Prophylaxis for the prevention of preterm labour must therefore be targeted, based on individual risk. The development and evaluation of such strategies should be an aim of research in this field.

1.4.6.3. *Antibiotics for the treatment of preterm labour*

Intrauterine infection threatens the well-being of the fetus and the mother. By the time labour is evident, inflammatory parturition pathways have already been activated, thus it seems unlikely that even vigorous treatment of a causative infection could arrest the progression of parturition at this stage. Results of a large randomized control trial in humans are consistent with this, showing no benefit of antibiotic use in the treatment of women presenting with spontaneous preterm labour (Kenyon, Taylor et al. 2001). Subsequent meta-analysis of this and other trials, also failed to show any neonatal benefits (King and Flenady 2002).

There does seem to be some benefit in the administration of antibiotics in cases of preterm rupture of membranes. Erythromycin use decreases the incidence of chorioamnionitis, prolongs the latency period to delivery and also lessens neonatal morbidities such as neonatal infection, positive blood culture, use of surfactant and oxygen therapy and abnormal cranial ultrasound on discharge (Kenyon, Boulvain et al. 2004). However, no effect on neonatal mortality is seen. It is possible that antibiotics in these cases prevent the sequelae of secondary, acute infections, which can evolve once the protective functions of the fetal membranes have been lost. It seems unlikely that they have any influence on the underlying mechanism of membrane rupture.

1.5. NATURAL ANTIMICROBIALS

Natural antimicrobials are genetically determined proteins that play a major part in both innate and adaptive immunity. They are an ancient form of host defence first established in lower phyla such as plants (thionins), insects (eg. cecropins, drosomycin and insect defensins) but now appreciated as being vital in the mammalian immune response. Over 800 natural antimicrobials have been recognized to date (http://bbcm1.univ.trieste.it/~tossi/pag1/htm) A single species will express a variety of different proteins, either constitutively or after induction by inflammatory stimuli, which can act synergistically to provide broad spectrum antimicrobial activity against bacteria, fungi and some viruses (Hancock and Diamond 2000). It is increasingly realized that many peptides also have other effects on a wide range of host functions (Bowdish, Davidson et al. 2006).

Typically natural antimicrobials are small cationic proteins composed of 12-45 amino acids containing excess basic lysine, arginine and histidine residues. They are divided into four structural classes based on 3-D structure; β-sheets, α-helices, extended peptides and loop peptides (Hancock and Diamond 2000). Their microbial killing may be effected via a variety of mechanisms including membrane depolarization, membrane permeablization, induction of hydrolases, disruption of membrane functions and/or damage of critical intracellular proteins (Zasloff 2002). Crucial to all these processes are selective interactions with the membranes of micro-organisms. Mammalian cell membranes are mainly constructed of zwitterionic phospholipids, which have no net charge overall, and any phospholipids with anionic head groups tend to face inwards. They are also stabilized by a high cholesterol content. In contrast, prokaryote membranes have many highly anionic phospholipids orientated outwards which interact with positively charged, hydrophobic antimicrobials. Natural antimicrobials form aggregates in the lipid bilayer, physically disrupting it, and, in some cases, allowing diffusion of peptides to intracellular targets (Matsuzaki 1999; Shai 1999; Yang, Weiss et al. 2000). Increasing ionic strength generally decreases natural antimicrobial activity, probably via reducing the strength of initial interactions.

Two major types of human natural antimicrobials are the human beta-defensins (HBDs) and two antileukoproteinases- secretory leukocyte protease inhibitor (SLPI) and elafin- which have "defensin-like" properties (Sallenave 2002).

1.5.1. Human beta-defensins (HBDs)

HBDs are members of the larger defensin family, which contain 28 – 42 amino acids which form triple-stranded β sheets on a frame of six disulphide linked cysteines. The classification into alpha (α) and beta (β) defensins depends on the position of the cysteine bonds, but they have very similar tertiary structures (Zimmermann, Legault et al. 1995). The α defensins (Human Neutrophil Peptides 1-4; HNP 1-4) are a product of neutrophils, forming 30-50% of the protein content of the azurophilic granules (Rice, Ganz et al. 1987). They are also found in the Paneth cells of the small intestine (Human Defensins 5 and 6; HD 5 and 6) (Selsted, Miller et al. 1992). A third class, the theta (θ) defensins, have been identified (Tang, Yuan et al. 1999), which have a circular structure, and enhanced antiviral activities (Lehrer 2004).

HBDs are predominantly epithelial products. The functions of HBD1-4 are the best characterized, although genomic data-mining has identified many additional defensin genes (Scheetz, Bartlett et al. 2002; Schutte, Mitros et al. 2002; Yamaguchi, Nagase et al. 2002). HBD1 was originally identified in an ultrafiltrate of human plasma from renal dialysis patients (Bensch, Raida et al. 1995). HBD2 and HBD3 were discovered in human skin, and the biological relevance of these factors was indicated by their particularly high expression in psoriatic plaques (Harder, Bartels et al. 1997; Harder, Bartels et al. 2001). HBD4 was identified by genomic screening techniques (Garcia, Krause et al. 2001).

It is now appreciated that HBD1, 2 and 3 are widely expressed, and a substantial amount of information regarding their functions has been gained from research of their production by skin, gut, respiratory and oral epithelia. HBD3 is also expressed in some non-epithelial tissues including skeletal and cardiac muscle and leukocytes (Garcia, Jaumann et al. 2001).

Unlike the HNPs, which are stored, HBD concentrations are generally governed by synthesis and secretion rates. Production of HBD1 is generally constitutive, whereas HBD2 and 3 tend to be upregulated by a variety of inflammatory stimuli including bacteria, bacterial products, and cytokines (Zhao, Wang et al. 1996; Harder, Bartels et al. 1997; Huttner and Bevins 1999; Mathews, Jia et al. 1999; O'Neil, Porter et al. 1999; Krisanaprakornkit, Kimball et al. 2000; Harder, Bartels et al. 2001; Chadebech, Goidin et al. 2003). The 5' flanking region of the HBD2 gene (*DEFB4*) contains NFκB, C/EBP and AP-1 binding sites (Tsutsumi-Ishii and Nagaoka 2002) whilst the region of the HBD3 gene (*DEFB103*) has C/EBP and AP-1 sites (Jia, Schutte et al. 2001). This suggests these elements are important in HBD expression. *In vivo*, HBD2 and HBD3 tend to be found in epithelia in association with infection and inflammation, whereas HBD1 is often intrinsically present in healthy tissues (Schonwetter, Stolzenberg et al. 1995; Hiratsuka, Nakazato et al. 1998; Liu, Wang et al. 1998; Singh, Jia et al. 1998; Milner, Cole et al. 2003; Wehkamp, Fellermann et al. 2005). However, HBD1 (*DEFB1*) gene expression may also have an inducible component, as it has recently been shown to be stimulated by IFN-γ *in vitro* (Joly, Organ et al. 2005). HBD4 (*DEFB104*) is also inducible, but has more restricted expression, found in testis, stomach, uterus, neutrophils, thyroid and kidney (Garcia, Krause et al. 2001).

Clusters of putative HBD genes have been found at 20p13, 20q11.1, and 6p12, but most HBDs are found in a cluster at 8p23.1, (Schutte, Mitros et al. 2002). This band is a frequent site of chromosomal rearrangements, and the cluster containing *DEFB4, DEFB103,* and *DEFB104* is polymorphic in copy number (Hollox, Armour et al. 2003). Individuals have 2–12 copies of this repeat per diploid genome. The copy number is reflected in mRNA expression levels, and recently Crohn's disease has been associated with both a decreased copy number of *DEFB4* and decreased intestinal mRNA expression of HBD2 (Fellermann, Stange et al. 2006). Copy number polymorphisms are not associated with severity of lung disease in cystic fibrosis (Hollox, Davies et al. 2005). It remains to be seen if the copy number correlates with other infectious or inflammatory conditions.

HBD1, 2 and 3 have strong activity against Gram-negative bacteria, being bactericidal at concentrations of 1-500µg/ml (Harder, Bartels et al. 1997; Schroder 1999; Harder, Bartels et al. 2001; Chen, Niyonsaba et al. 2005). HBD2 and HBD3 also have activity against yeasts such as *Candida albicans* (Harder, Bartels et al. 1997; Harder, Bartels et al. 2001). There is conflicting data on the effects of defensins on Gram-positive organisms. Earlier studies suggested HBD2 had only bacteriostatic activity, at concentrations greater than 100µg/ml (Harder, Bartels et al. 1997). Recent reports have shown HBD1, 2 and HBD3 are all lethal to a range of Gram-positive organisms at concentrations as low as 10µg/ml (Chen, Niyonsaba et al. 2005) including, in the case of HBD3, multi-drug resistant strains (Harder, Bartels et al. 2001). These effects are synergistic with each other and other classes of natural antimicrobial such as the cathelicidin LL-37, and lysozyme (Chen, Niyonsaba et al. 2005). Interestingly, this study found that HBDs have enhanced antimicrobial efficiency at low pH (4.6), which had not been shown before (Valore, Park et al. 1998).

HBDs have a number of functions in addition to their antimicrobial activity, many of which involve regulation of the inflammatory and immune responses. HBD2 induces histamine release and stimulates prostaglandin D_2 production by rat mast cells (Niyonsaba, Hirata et al. 2003). HBD2 (Niyonsaba, Iwabuchi et al. 2002) 3 and 4 (Chen, Niyonsaba et al. 2007) are also chemoattractants for mast cells. The mouse orthologue of HBD2, mBD2, is an endogenous ligand for TLR4 on immature dendritic cells and induces dendritic cell maturation (Biragyn, Ruffini et al. 2002). In humans, HBD2 is chemotactic for dendritic cells and T-cells (Yang, Chertov et al. 1999) and TNFα treated neutrophils (Niyonsaba, Ogawa et al. 2004) apparently by interaction with the CCR6 receptor. Recent studies have shown that HBD2, 3, and 4 stimulate production of a variety of chemokines and cytokines (IL-8, IL-6, IL-18, IL-20 IL-10, CCL2, CCL20 and CCL5) from human keratinocytes and also increased keratinocyte migration, and proliferation in an EGFR/signal transducer and activator of transcription (STAT) dependent way (Niyonsaba, Ushio et al. 2005; Niyonsaba, Ushio et al. 2007).

1.5.2. The antileukoproteinases- SLPI and elafin

Secretory leukocyte protease inhibitor (SLPI) and elafin are members of the four-disulphide core family of antiproteinases (Schalkwijk, Wiedow et al. 1999). These are produced locally at sites of injury and counteract the effects of excess proteolytic enzymes released by inflammatory cells. They also have direct antimicrobial activities (Hiemstra, Maassen et al. 1996; Simpson, Maxwell et al. 1999) and can regulate the innate and adaptive immune responses (Fitch, Roghanian et al. 2006).

1.5.2.1. *Structure and distribution*

SLPI

SLPI is an 11.7 kDa protein, consisting of 107 amino acids including 16 cysteine residues which form eight disulphide bridges (Seemuller, Arnhold et al. 1986). It is expressed constitutively by many epithelia, including the mucosa of the respiratory, intestinal and genital tracts and oral cavity (Franken, Meijer et al. 1989; Sallenave, Silva et al. 1993; Si-Tahar, Merlin et al. 2000; Saitoh, Masuda et al. 2001) as well as neutrophils, macrophages and mast cells (Bohm, Aigner et al. 1992; Jin, Nathan et al. 1997; Sallenave, Si Tahar et al. 1997; Westin, Polling et al. 1999).

The SLPI gene is localized on chromosome 20q12-13. 2 (Kikuchi, Abe et al. 1997) and is a non-polymorphic, stable gene but can be modulated at both the transcriptional and translational levels (Maruyama, Hay et al. 1994). The promoter region has not been extensively studied, but early investigations demonstrated an AP-1 and AP-2 consensus site (Abe, Kobayashi et al. 1991). Epithelial expression of SLPI expression appears to be tissue specific. It is increased by a variety of inflammatory stimuli including LPS, IL-1β, TNFα, EGF, HNPs and human neutrophil elastase whilst anti-inflammatory mediators such as TGFβ and IL-10 can decrease production (Williams, Brown et al. 2006).

Elafin

Elafin is a 9.9 kDa protein composed of 95 amino acids and shows 40% sequence identity with the SLPI molecule (Sallenave and Silva 1993). Its C-terminal domain

contains an antiproteinase site which is similar to that of SLPI. The N-terminal contains characteristic sequences which allow it to be crosslinked into polymers or bind to extracellular matrix via transglutamination (Nara, Ito et al. 1994). There is some evidence that tissue bound elafin is more effective at preventing elastase mediated tissue damage *in vivo* (Tremblay, Vachon et al. 2002).

Elafin was first identified in bronchial mucus (Hochstrasser, Albrecht et al. 1981) (Sallenave and Ryle 1991) and has been subsequently found in skin (Wiedow, Schroder et al. 1990), intestine (Schmid, Fellermann et al. 2007) and endometrium (King, Critchley et al. 2003). It may be constitutively expressed at a low level, but expression is greatly induced by IL-1β and TNFα (Sallenave, Shulmann et al. 1994; Tanaka, Fujioka et al. 2000). The gene for elafin has 5' regulatory sites for NFκB and AP-1(Zhang, Zou et al. 1995), and the response to cytokines appears to involve MAPK pathways in the skin (Pfundt, Wingens et al. 2000) and NFκB pathways in the lung (Bingle, Tetley et al. 2001).

1.5.2.2. *Antiprotease activity*

SLPI can inhibit a variety of human neutrophil proteases including cathepsin G, trypsin, chymotrypsin and chymase, however its greatest activity seems to be against neutrophil elastase (Thompson and Ohlsson 1986; Boudier and Bieth 1992). Elafin has more restricted antiprotease activity to SLPI, inhibiting porcine pancreatic elastase, human neutrophil elastase and proteinase-3 (Schalkwijk, Wiedow et al. 1999).

1.5.2.3. *Antimicrobial activity*

Both SLPI and elafin have direct antimicrobial activity against Gram-negative and Gram-positive organisms, but elafin is the more effective agent (Simpson, Maxwell et al. 1999). In SLPI, bactericidal activity is confined to the N-terminal (Hiemstra, Maassen et al. 1996), whereas the antibacterial activity of elafin is found in both the C- and N-domains, and the full length protein is required for the most efficient activity (Simpson, Maxwell et al. 1999).

SLPI has *in vitro* bactericidal activity against *Escherichia coli* and *Staphylococcus aureus* (Hiemstra, Maassen et al. 1996), *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, (Wiedow, Harder et al. 1998) *Salmonella typhimurium* (Si-Tahar, Merlin et al. 2000) and Group A Streptococcus (Fernie-King, Seilly et al. 2002). In lung fluid the antibacterial properties of SLPI are synergistic with lactoferrin and lysozyme, but additive with HBDs and the cathelicidin LL-37 (Singh, Tack et al. 2000). SLPI is also antifungal against *candida* and *aspergillus spp* (Tomee, Hiemstra et al. 1997; Wiedow, Harder et al. 1998), and specific anti-HIV activities have been demonstrated *in vitro* (McNeely, Dealy et al. 1995; McNeely, Shugars et al. 1997; Shine, Wang et al. 2002). *In vivo*, lower concentrations of SLPI in vaginal fluid and saliva have been associated with increased vertical and breastmilk HIV transmission (Pillay, Coutsoudis et al. 2001; Farquhar, VanCott et al. 2002).

Elafin has *in vitro* antimicrobial activity against the Gram-negative respiratory pathogen *Pseudomonas aeruginosa* and Gram-positive *Staphyloccus aureus* (Simpson, Maxwell et al. 1999; McMichael, Maxwell et al. 2005). Cell free supernatants of *Pseudomonas aeruginosa* also stimulate elafin production by keratinocytes, suggesting the biological relevance of these findings (Meyer-Hoffert, Wichmann et al. 2003). In addition, *in vivo* antimicrobial effects have been demonstrated in mice which express the human elafin secondary to adenoviral gene transfer. These show enhanced bacterial elimination and decreased bacterial haematogenous spread in response to challenge with *Pseudomonas aeruginosa* (Simpson, Wallace et al. 2001).

The mechanism of antileukoproteinase antimicrobial activity is unclear. They are cationic proteins, thus may interact with microbial membranes in a defensin like manner. Like defensins, their bactericidal effects are diminished by high salt concentrations (Hiemstra, Maassen et al. 1996).

1.5.2.4. *Immunomodulatory activity*

SLPI and elafin demonstrate a variety of anti-inflammatory effects in a number of tissues which cannot be solely attributed to their antiproteinase or antibacterial activities. In an animal model of chemically induced lung fibrosis, administration of SLPI decreases tissue damage, and even a truncated form which lacks the anti-elastase domain, exerts this effect (Mitsuhashi, Asano et al. 1996). SLPI also decreases cortical damage after ischaemic induced stroke in rats (Wang, Li et al. 2003). Forced expression of elafin reduces inflammation in animal models of atherosclerosis (Henriksen, Hitt et al. 2004) and myocardial infarction (Ohta, Nakajima et al. 2004). In humans, SLPI and elafin induction appear attenuated in Crohn's disease, characterized by inflammation affecting the full thickness of intestinal wall, when compared to ulcerative colitis, which is an inflammatory condition only involving the mucosa (Schmid, Fellermann et al. 2007).

These effects may be mediated in three ways – by inhibition of inflammatory cell infiltration, by inhibiting release of inflammatory mediators, and/or by promotion of resolution of inflammation (Williams, Brown et al. 2006). SLPI has been shown to decrease neutrophil recruitment in a model of arthritis (Sehnert, Cavcic et al. 2004) whilst elafin reduces neutrophil infiltration in LPS induced lung injury (Vachon, Bourbonnais et al. 2002). SLPI can also inhibit histamine release from mast cells (Dietze, Sommerhoff et al. 1990; He, Xie et al. 2004) and suppress eosinophil degranulation (Murata, Sharmin et al. 2003). Both SLPI and elafin may promote phagocytosis of apoptotic cells. Elafin inhibits neutrophil elastase cleavage of the CD14 from macrophages which promotes recognition of apoptotic cells (Henriksen, Devitt et al. 2004). SLPI production by macrophages is increased during phagocytosis (Odaka, Mizuochi et al. 2003), and SLPI also stimulates production of anti-inflammatory cytokines TGFβ and IL-10 (Sano, Shimizu et al. 2000).

Some of the anti-inflammatory effects of the antileukoproteianses may involve inhibition of NFκB. *In vitro*, overexpression of SLPI suppresses NFκB activation (Jin, Nathan et al. 1997; Sano, Shimizu et al. 2003; Henriksen, Hitt et al. 2004). It can prevent proteosome dependent IκBβ degradation (Lentsch, Jordan et al. 1999)

and also directly bind to NFκB consensus sites on DNA, inhibiting transcription (Taggart, Cryan et al. 2005). SLPI also inhibit LPS binding to CD14 and inhibits TLR signalling (Ding, Thieblemont et al. 1999).

Studies involving the expression of human elafin in mice have reported that antileukoproteinases also have some proinflammatory activities. Forced elafin expression upregulates TNFα expression in response to LPS in murine macrophages (McMichael, Roghanian et al. 2005), whilst mice expressing elafin show increased inflammatory infiltration in response to LPS (Simpson, Cunningham et al. 2001; Sallenave, Cunningham et al. 2003). It is appreciated that adenovirus vectors may themselves have some effect on the response. In addition, the conflicting results between these, and studies using recombinant elafin (Vachon, Bourbonnais et al. 2002) may be explained by additional effects of transgenic elafin on intracellular pathways. Serum TNFα levels in these animals are paradoxically lower, supporting the theory that elafin may stimulate local innate immune responses whilst downregulating systemic inflammation (Sallenave, Cunningham et al. 2003; Williams, Brown et al. 2006).

Recent studies have shown that the antileukoproteinases also interact with the adaptive immune response. Elafin increases recruitment of dendritic cells in murine lungs exposed to adenovirus-antigen, and promotes a type-1 response evidenced by increased production of IFNγ and TNFα. (Roghanian, Williams et al. 2006; Williams, Brown et al. 2006).

Wound healing and tissue remodelling are other processes involving the antileukoproteinases. SLPI and elafin are produced in response to cutaneous injury (van Bergen, Andriessen et al. 1996; Wingens, van Bergen et al. 1998) and SLPI null mice show impaired cutaneous wound healing and increased inflammation and elastase activity (Ashcroft, Lei et al. 2000; Angelov, Moutsopoulos et al. 2004). SLPI can also modulate production of matrix metalloproteinases from monocytes by inhibiting enzymes involved in PGE_2 synthesis (Zhang, DeWitt et al. 1997).

1.5.3. Natural antimicrobials in the reproductive tract

Expression of natural antimicrobial RNA and protein by maternal and fetal tissues in pregnancy are summarized in Figures 1.3 and 1.4.

1.5.3.1. *α-defensins*

α-defensins are localized in endometrial neutrophils (King, Critchley et al. 2003) and expression of HD5 in the endometrium and cervicovaginal lavages is cyclical (Quayle, Porter et al. 1998). In the non-pregnant state HD5 has also been detected in the vagina, ectocervix, endocervix, endometrium and fallopian tubes (Svinarich, Wolf et al. 1997; Quayle, Porter et al. 1998) and HNP 1-3 and HD5 are found in cervicovaginal secretions (Valore, Wiley et al. 2006).

In pregnancy HNPs are detectable in the cervicovaginal fluid of approximately 70% of pregnant women at 24-29 weeks gestation, and levels positively correlate with intermediate bacterial vaginosis status, but not bacterial vaginosis itself (Balu, Savitz et al. 2002). Women with elevated concentrations of cervicovaginal HNPs at 24-29 weeks also had a greater risk of delivering before 32 weeks (Balu, Savitz et al. 2003). However, as HNPs are neutrophil granule products, this elevation may simply reflect the neutrophil infiltration that is known to occur in preterm labour, rather than actually being involved in the pathogenesis of the condition. mRNA corresponding to HNP 1 or 3 had been found in amnion, chorion and placental tissue (Svinarich, Gomez et al. 1997), and HD5 mRNA was also found in samples from the chorion and cervix (Svinarich, Wolf et al. 1997) but their cellular location was not determined.

1.5.3.2. *HBDs*

HBD 1-4 are produced by the endometrium, and their expression varies with the menstrual cycle (Fleming, King et al. 2003; King, Critchley et al. 2003; King, Fleming et al. 2003). This suggests they may be important in defence at vulnerable times such as menstruation and implantation. HBD1 is expressed in vaginal epithelium (Valore, Park et al. 1998) and HBD2 is produced by the immortalized

human vaginal cell line PK E6/E7 (Pivarcsi, Nagy et al. 2005). In these cells expression is significantly upregulated by the microbial products LPS, PG and heat-killed *Candida albicans*. In contrast, vaginal expression of the mouse beta-defensins 1, 2 and 4 are not affected by bacterial products (Soboll, Schaefer et al. 2006). HBD1 and 2 are present in vaginal secretions in non-pregnant women, and levels are decreased in association with bacterial vaginosis (Valore, Park et al. 2002; Valore, Wiley et al. 2006).

At the outset of this project there was little published data on the expression of HBDs in pregnancy, except for an abstract suggesting HBD1 and 2 mRNA is expressed in the placenta and chorion, but not the amnion (Feng, Pan et al. 2003). Since then HBD1, 2 and 3 expression in both fetal membranes and placenta has been confirmed by immunohistochemistry (King, Paltoo et al. 2007). In addition, this study showed HBD2 production by the chorion and placental trophoblast that was responsive to IL-1β. One other paper has provided further immunohistochemical evidence of HBD3 expression in the amnion (Buhimschi, Jabr et al. 2004).

1.5.3.3. *SLPI and elafin*

SLPI is secreted by the endometrium, and again expression is cyclical (Fleming, King et al. 2003; King, Critchley et al. 2003; King, Critchley et al. 2003). Levels are also influenced by the levonorgestrel intra-uterine system and the combined oral contraceptive pill, suggesting hormonal regulation (Fleming, King et al. 2003). Interestingly, progesterone has been shown to influence SLPI, but not elafin expression in a cell line expressing the progesterone receptor (King, Morgan et al. 2003).

In both pregnant and non-pregnant women cervicovaginal concentrations of SLPI are decreased in the presence of genital tract infections, including bacterial vaginosis (Draper, Landers et al. 2000; Valore, Wiley et al. 2006). SLPI is expressed in the vagina in higher levels than in uterine tissues in the mouse, but production is unaffected by bacterial products or TLR agonists (Soboll, Schaefer et al. 2006). SLPI is also constitutively produced by both vaginal and cervical immortalized human cell

lines (VK E6/E7, ECT E6/E7 and END E6/E7), but secretion is not increased by treatment with the cytokines TNFα or IFNγ (Fichorova and Anderson 1999).

SLPI is found in the amniotic fluid and levels increase during pregnancy (Zhang, Shimoya et al. 2001) and at the onset of labour (Denison, Kelly et al. 1999). Decreased levels of SLPI have also been reported in association with prelabour rupture of membranes occurring in the absence of infection (Helmig, Romero et al. 2002). Amnion, chorion and placental secretion of SLPI has been demonstrated (Denison, Kelly et al. 1999) and it is also produced by first trimester decidua (King, Critchley et al. 2000). Production in the amnion may be stimulated by IL-1β and TNFα (Zhang, Shimoya et al. 2001). In addition, SLPI was found in extremely high levels in the cervical mucus plug in pregnancy, where it might be important in microbial defence and protection against protease mediated fetal membrane damage (Helmig, Uldbjerg et al. 1995). PGE_2 inhibits its production by the cervix (Denison, Calder et al. 1999).

Elafin has been immunolocalized in the vagina (Pfundt, Wingens et al. 2000). It has recently also been shown in the placenta and fetal membranes and is secreted by trophoblast in response to IL-1β (King, Paltoo et al. 2007).

1.5.3.4. *Other antimicrobials*

A number of other natural antimicrobial factors including lysozyme, lactoferrin, LL-37, Bacteriacidal Permeability Inducing Factor (BPI) and calprotectin have been identified in the amniotic fluid and vernix, which may have a role in protection of the fetus in pregnancy (Figure 1.4).

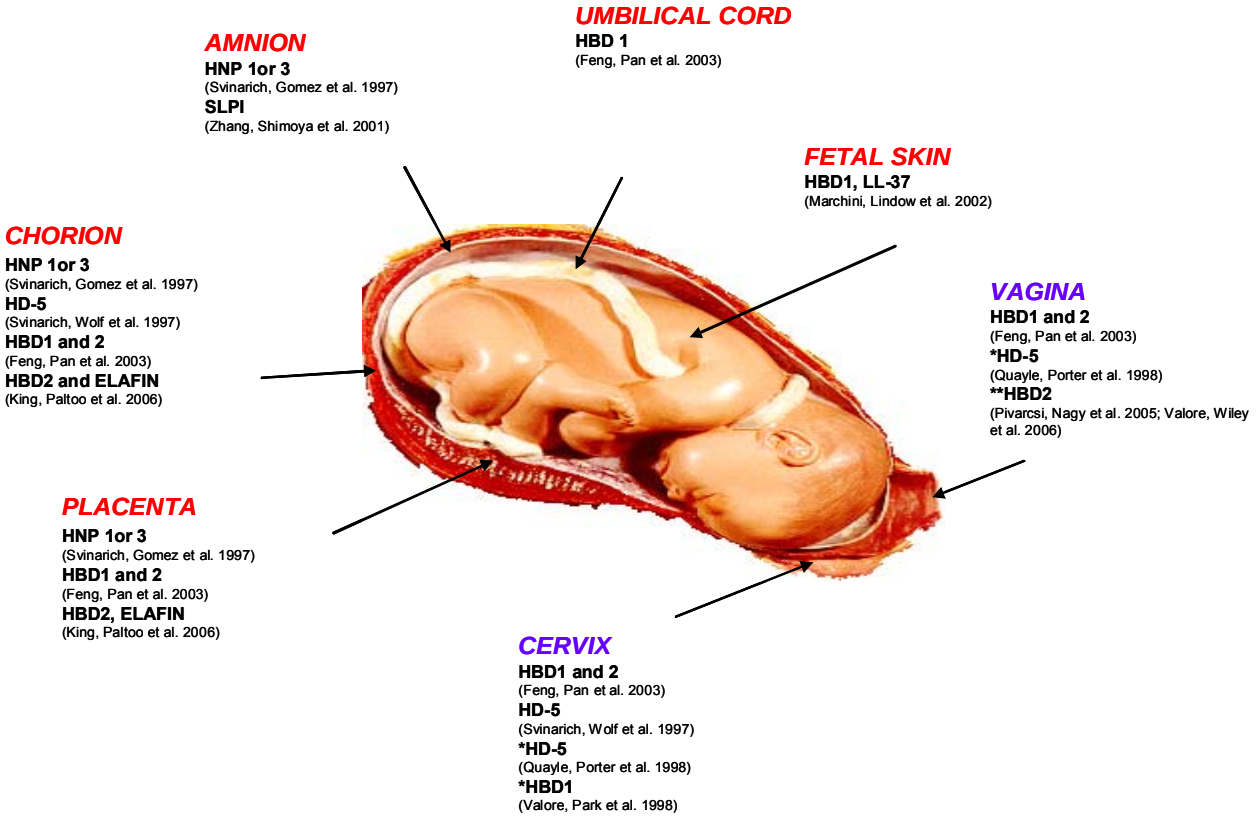


Figure 1.3

Fetal and Maternal natural antimicrobial RNA expression in pregnancy.

* expression identified in non-pregnant reproductive tract. ** expression in immortalized cell line.

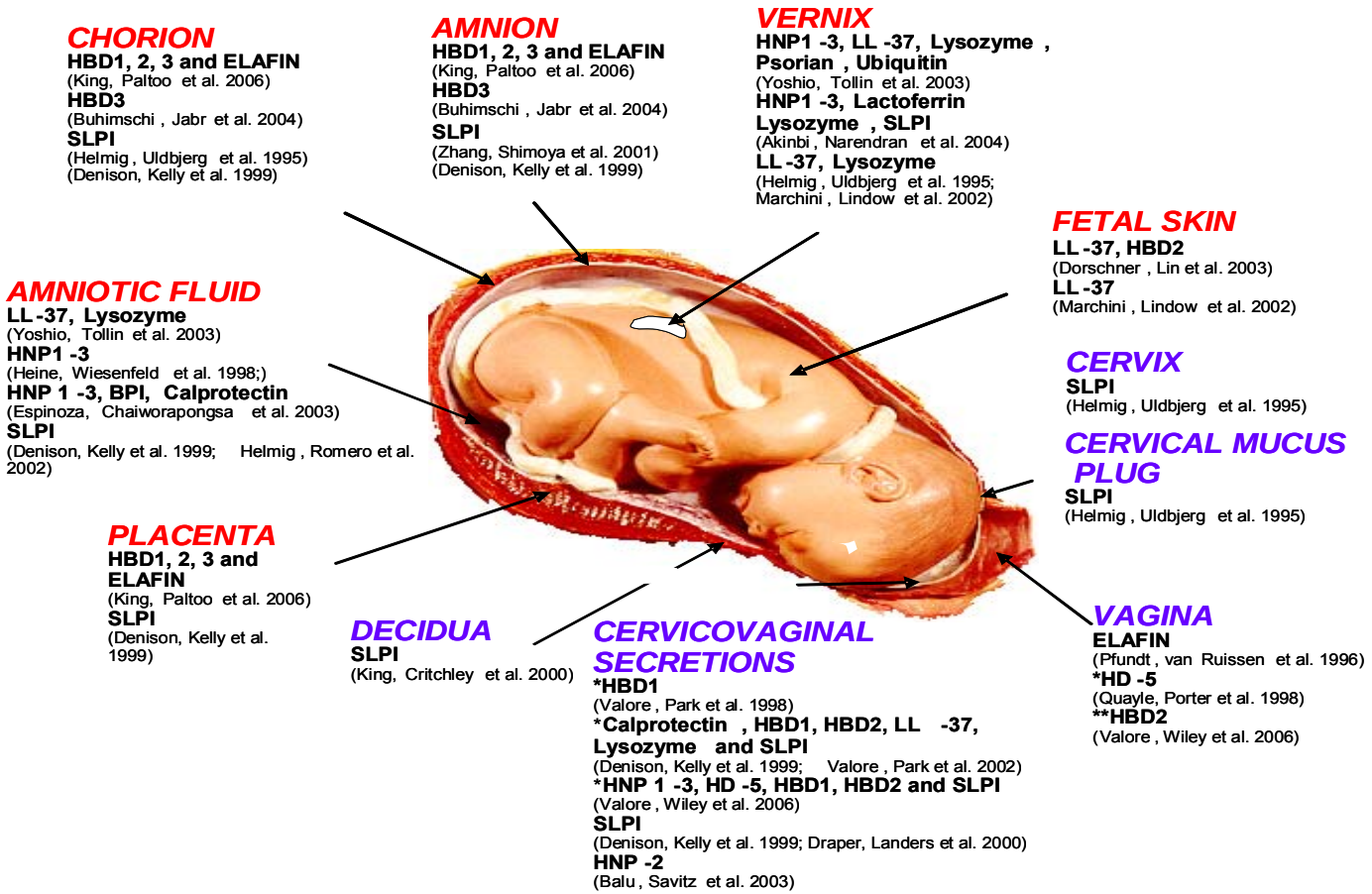


Figure 1.4

Fetal and Maternal natural antimicrobial proteins in pregnancy.

*identified in non-pregnant reproductive tract. ** identified in immortalized cell line.

1.6. SUMMARY

This thesis concerns aspects of the innate immune response which is a complex process involving cytokines, chemokines and natural antimicrobials, briefly outlined above. The innate immune response provides our first line of defence against infection and is responsible for the inflammation seen in response to injury. However, it is increasingly recognised that inflammation is also involved in physiological processes, including that of parturition. Normal labour is associated with an inflammatory response, but this is exaggerated in case of preterm labour. Elucidation of this process is vital, because preterm labour remains the leading cause of perinatal morbidity and mortality and rates of this pregnancy complication seem to be increasing.

Natural antimicrobials are a relatively under researched component of the innate immune response in pregnancy. Nevertheless, they may be important in the prevention of infections which are associated with approximately one third of cases of preterm labour. In addition, natural antimicrobials are involved in a variety of biological processes relevant to parturition, including chemotaxis, tissue remodelling and protease inhibition. Their production in pregnancy is the subject of the studies described below.

1.7. HYPOTHESIS AND AIMS

This project was based on the hypothesis that natural antimicrobials are produced by epithelia of the reproductive tract in pregnancy. The natural antimicrobials studied were the HBDs, SLPI and elafin. They were chosen because they are epithelially produced proteins involved in the innate immune system of other organ systems. Their production is often affected by bacterial microflora, bacterial wall products such as LPS and LTA, and inflammatory cytokines such as IL-1β and IL-17. It was hypothesized that these factors may also influence natural antimicrobial production in the reproductive tract in pregnancy.

Two areas were focused on – the lower genital tract (cervix and vagina) and the amnion. The lower genital tract was chosen as this area is host to a diverse microflora and frequently exposed to pathogens, so would seem to have an effective innate immune system. The amnion is the innermost fetal membrane. Although it is the last to be affected by ascending infection, it is the inflammatory response of this tissue which is crucial in the onset of labour. Natural antimicrobials may be a component of this.

The principal aims of the studies in the lower genital tract were

- To establish if HBDs, SLPI and elafin are present in cervicovaginal secretions in pregnancy.
- To investigate whether levels of HBDs, SLPI and elafin in cervicovaginal secretions vary in association with bacterial vaginosis, a condition where there is abnormal vaginal flora.
- To examine HBD, SLPI and elafin expression in vaginal, ectocervical and endocervical epithelial cell lines, and the effect of LPS, LTA and IL-1β on production.

The principal aims of the studies in the amnion were

- To determine whether HBDs, SLPI and elafin are expressed by the amnion.
- To examine the effect of labour on the expression of HBDs, SLPI and elafin in the amnion.
- To investigate the effect of the inflammatory cytokines on the production of HBDs, SLPI and elafin in the amnion, and explore pathways regulating their expression.

2. General Methods

All materials, reagents and cell lines used are detailed in Appendix 1.
Recipes for reagents are provided in Appendix 2.

2.1. SAMPLE COLLECTION

2.1.1. Ethical approval and consent

Approval from the Lothian Local Research Ethics Committee (REC: Reference number 04/S1101/24) was granted for all experimental work described in this thesis. Written information was provided and informed consent was obtained from participants who donated biological samples (Appendix 3).

2.1.2. Amnion, choriodecidua and placenta

Amnion was collected from a total of 40 women undergoing prelabour elective Caesarean section at 38-39 weeks and seven women who had a spontaneous vertex delivery (SVD) at 38-40 weeks. Indications for Caesarean section were breech presentation, previous Caesarean section or maternal request. In three prelabour cases choriodecidua and placenta were also collected. This study was designed to examine natural antimicrobial production in normal low risk pregnancies. All women were therefore healthy with no medical conditions and a normal BMI. They had uncomplicated singleton pregnancies, with no signs of infection (pyrexia, history of prolonged rupture of membranes, uterine tenderness or irritability). Women were excluded if they were under 16 years old, or had positive serology for HIV or Hepatitis B. In addition, samples were discarded if meconium stained liquor was found or there was a placental abnormality on gross inspection.

Amnion for explant culture and primary cell culture was stripped in its entirety from underlying tissue, up to the origin of the umbilical cord, rinsed and transported back to the laboratory in sterile phosphate buffered saline (PBS).

Fetal membranes for RNA extraction were collected within 5 minutes of delivery. Amnion was stripped from the underlying choriodecidua and each tissue washed thoroughly in PBS. Samples were taken from sites at least 5 cm from the site of membrane rupture or placental edge, and areas with adherent blood were avoided. Preliminary experiments showed that two 12mm discs of chorion and three 12mm discs of amnion provided optimal concentrations of RNA after extraction. Discs were cut from the membrane using a sterile cork borer and submerged in 2ml RNAlater. Samples were taken in triplicate, stored at -80°C and processed in batches within 21 days. Full thickness placental samples, approximately 0.5cm^2, were taken in duplicate from central cotyledons, and placed in 5ml of RNAlater, before storage as above.

Samples of whole fetal membranes were collected for histological assessment and immunohistochemistry. Strips of full thickness membrane, approximately 0.5cm wide, extending from the site of membrane rupture to the placental edge were cut and fixed in 10% neutral buffered formalin overnight at 4°C. They were then rinsed in 70% ethanol, rolled and embedded in paraffin.

2.1.3. Endometrium

RNA from endometrium was used as a positive control for natural antimicrobials, kindly supplied by Professor Hilary Critchley (LREC/1702/94/6/17). As individual natural antimicrobials are differentially expressed throughout the menstrual cycle, endometrium was used from the period of maximal expression for each natural antimicrobial (Menstrual for elafin and HBD2; Proliferative for HBD4; Mid-secretory for HBD1, HBD3 and SLPI (King, Critchley et al. 2003)).

Samples were collected from women undergoing gynaecological procedures for benign conditions. All had regular menstrual cycles (28-35 days) and had not received any hormonal treatments for 3 months prior to biopsy collection. Menstrual cycle stage was determined from the date of the patient's last menstrual period (LMP), circulating serum oestradiol and progesterone concentrations and histological

dating. Endometrial biopsies were immersed in Tri-reagent for RNA extraction. A portion was also fixed in 10% neutral buffered formalin overnight at 4 °C, stored in ethanol and then wax embedded for subsequent histological examination, which was normal in all cases.

2.1.4. Cervicovaginal secretions

Cervicovaginal secretions were obtained from women (n=113), with uncomplicated singleton pregnancies attending community antenatal clinics in the second trimester of pregnancy (12-20 weeks gestation). The collection method is detailed in chapter 3.

2.2. *IN VITRO* CULTURE

All cells and tissues were maintained at 37°C in 5%CO_2 and 95% air in humidified conditions.

2.2.1. Cell culture

2.2.1.1. *Primary Amnion Epithelial Cells*

Amnion epithelial cells were cultured using a method provided by Professor P Bennett's group at Imperial College, London (Bennett, Rose et al. 1987). Amnion was washed in sterile PBS, cut into strips, approximately 5 cm long and steeped in 0.5mM EDTA (Ethylenediaminetetraacetic acid) at room temperature for 15 minutes. Cells were dissociated by incubation in 100ml of Dispase solution (2.5mg/ml) at 37°C for 45 minutes. The strips of tissue were removed to 60 ml of Rosewell Park Memorial Institute Medium (RPMI 1640) culture medium, and cells detached by vigorously shaking for 90 seconds. This process was repeated in a further 40ml of RPMI, the solutions combined and centrifuged for 5 minutes at 8,000g. Cells were resuspended at a concentration of 1.5-2.0 x 10^6 per ml in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50µg/ml) streptomycin (50µg/ml) gentamycin (20µg/ml) and L-glutamine (2mM). Viability was assessed by trypan blue exclusion. 2.5ml of cell suspension per well was plated in 6 well plates.

After 24 hours of culture, cells were washed twice and fresh medium added. Cells usually achieved confluence within a further 24 hours, whereupon medium was changed to serum-depleted (2% FCS) for 20 hours before treatments were added. 2% FCS was used because preliminary experiments showed decreased cell viability after 48 hours of culture in the absence of serum. Treatments are detailed in Chapters 4 and 5. Unless otherwise stated, treatments were applied in quadruplicate, with one set of replicates used for RNA extraction, and one set used for protein analysis.

Epithelial origin of primary cultured cells was confirmed by immunocytochemistry, with over 95% of cells positive for the epithelial cell marker pancytokeratin (section 2.5.2).

2.2.1.2. *WISH Cells, FL Cells and He-La Cells*

FL, WISH and He-La cells were plated at a density of $0.5x10^6$cells/ml (2.5 ml per well) in 6 well plates. They were otherwise cultured in a manner identical to primary amnion cells.

2.2.1.3. *VK2 E6/E7, ECT E6/E7 and END E6/E7 cells*

VK2 E6/E7, ECT E6/E7 and END E6/E7 cells were cultured in keratinocyte serum free media, supplemented with 0.1ng/ml human recombinant epidermal growth factor (EGF), 0.05 mg/ml bovine pituitary extract (BPE) and 44.1mg/L calcium chloride (to give a final concentration of 0.4mM). They were plated at a density of $0.5x10^6$cells/ml (2.5 ml per well) in 6 well plates.

2.2.2. Amnion explant culture

Amnion for explant culture was washed in PBS, and adherent blood clot carefully removed. 12mm discs were cut using a cork borer and placed in 12 well plates in 1.5 mls of RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50µg/ml) streptomycin (50µg/ml) gentamycin (20µg/ml) and L-glutamine (2mM). The following day tissue was gently washed and serum-depleted (2% FCS) medium added before a further 20 hours of culture. Treatments were added as detailed in

Chapter 4. Following treatment, amnion was removed to RNA storage solution (RNAlater), and stored at -80°C for subsequent RNA extraction. Three discs of tissue were used for each RNA extraction; therefore each treatment was applied to six wells to provide duplicate samples. Media from each set of three discs was combined and stored at -20°C for ELISA.

2.3. RNA EXTRACTION AND REVERSE TRANSCRIPTION

Real-time PCR allows quantification of transcription levels of specific genes, relative to the amount in a control sample. This technique was used to examine natural antimicrobial mRNA cultured cells and tissue explants.

2.3.1. RNA extraction

RNA was extracted using the RNeasy mini system which uses centrifuge columns containing silica-gel membranes to selectively bind RNA. Salt and ethanol containing buffers are then applied, to wash away contaminants, and the RNA eluted with water.

Separate protocols were used for extraction from cells and tissue. Cultured cells were denatured in RLT buffer which contains guanidine isothiocyanate (GITC) to inhibit RNase activity, and homogenized by repetitive (>6 times) passage through an 18-gauge needle and syringe. Amnion tissue is tough and elastic, and was more difficult to lyse efficiently. Preliminary experiments found that homogenization in tri-reagent, an extraction agent containing phenol and GITC, using the TissueLyser (Qiagen), gave optimal RNA yields. A DNA digestion was also required, to remove genomic DNA contamination. The protocols are detailed below.

2.3.1.1. *RNA extraction from cultured cells*

Cells were denatured with 350µl per well of RLT buffer and passed through an 18-gauge needle 6 times. Equal volumes of 70% ethanol were then added to each sample and mixed by inversion. This mixture was applied to RNeasy mini spin columns, and

centrifuged for 15 seconds at 8,000g at room temperature, whereupon the lysate was discarded. 700µl of RW1 buffer was added to each tube, and centrifuged for another 15 seconds. The mini-spin columns were then transferred to fresh collection tubes, and washed twice with 500µl of RPE buffer. They underwent a 2 minute and a 1 minute centrifugation, to ensure all traces of ethanol were removed, which could interfere with downstream reactions. RNA was eluted using 50µl of RNase free water. RNA was stored at -80°C.

2.3.1.2. *RNA extraction from explants and tissue*

Samples (which had been frozen in RNA storage buffer- RNAlater) were thawed, and removed to a secure-lock eppendorf with 1 ml of tri-reagent and a 5 mm stainless steel bead. Samples were agitated by the TissueLyser for four 3 minute cycles at 30 Hz.

RNA was precipitated by the addition of 200µl of chloroform, mixed thoroughly and transferred to Eppendorf phase lock tubes. Centrifugation at 18,000g for 15 minutes at 4°C separated the homogenate into an aqueous and an organic phase, with RNA being retained in the aqueous phase. This was removed to a clean tube, and an equal volume of ethanol added and mixed by inversion. The mixture was then applied to an RNeasy mini-column, and centrifuged for 15 seconds at 8,000g at room temperature. The lysate was discarded, 350µl of RW1 buffer added, and the column spun again. DNase I was diluted in buffer, and applied directly to the silica membrane, and incubated at room temperature for 15 minutes. The column was washed with a further 350µl of RW1 buffer. The mini-spin columns were then transferred to fresh collection tubes, and washed twice with 500µl of RPE buffer. They underwent a 2 minute and a 1 minute centrifugation, to ensure all traces of ethanol were removed. RNA was eluted using 50µl of RNase free water. RNA was stored at -80°C.

2.3.2. RNA integrity and measurement

2.3.2.1. *Agilent Bioanalysis*

RNA quality was checked by the Agilent 2100 Bioanalyser system in combination with RNA_{6000}nano chips. This uses microcapillary electrophoresis to determine the concentration and integrity of RNA, and shows the percentage of ribosomal impurities present. Agilent software allows calculation of the RNA integrity number (RIN), a standardized means of expressing RNA integrity (Schroeder, Mueller et al. 2006).

RNA obtained from tissue was found to vary in quality and quantity, thus all tissue-derived RNA was assessed using the Agilent Bioanalyzer. Only samples with intact 18S and 28S peaks were reverse transcribed.

RNA obtained from cultured cells was of a consistently high standard, with RIN of >8 in the initial 60 samples analyzed (Figure 2.1). Subsequent samples were therefore presumed to be intact, and were only quantified and assessed for purity by spectrophotometry.

2.3.2.2. *Spectrophotometry*

RNA optical density was measured at 260 and 280nm, to allow calculation of the concentration and purity of the sample, using the Genequant RNA/DNA calculator or the Nanodrop ND-1000 spectrophotometer. A 260:280 value of around 1.8 indicates a pure RNA sample. Ratios of 1.7 to 2.1 were taken to be acceptable. RNA was stored at –80°C for further use.

2.3.3. Reverse Transcription

200ng template RNA was reverse transcribed using random primers with $MgCl_2$ (5.5mM), dNTPs (1mM), random hexamers (2.5µM), RNAase inhibitor (0.4IU/µl), and multiscribe reverse transcriptase (1.25IU/µl), in a total volume of 10µl. Mineral oil was added to prevent evaporation, and samples were incubated for 60 minutes at

25°C, 45 minutes at 48°C and then at 95°C for 5 minutes. Resultant complementary DNA (cDNA) was diluted 2.5 fold with TE buffer and stored at –20 °C until further use.

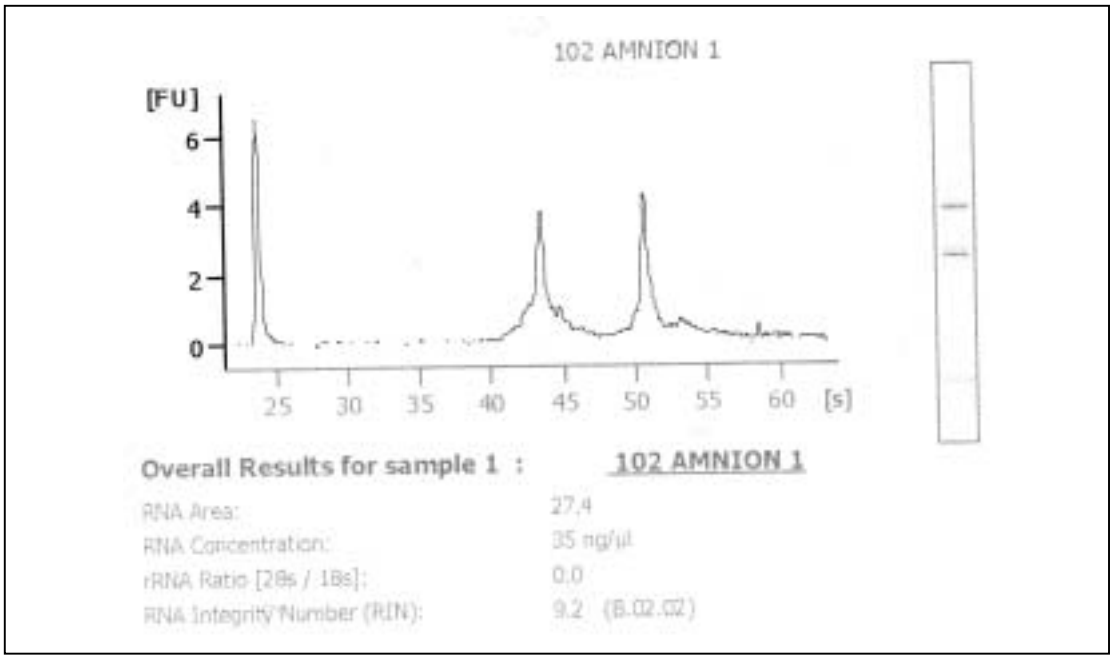


Figure 2.1

Scanned results of RNA from amnion explant analyzed by the Agilent Bioanalyser. The graph represents the fluorescence of the 18S and 28S fragments, from which the concentration is calculated. The 28S:18S ratio indicates the purity of the sample, with values approximating 2 being pure. The electropherogram is a graphical representation of the integrity of the sample, whereas the RIN number is a standardized means of expressing RNA integrity, from 0-10.

2.4. TAQMAN QUANTITATIVE POLYMERASE CHAIN REACTION

Taqman quantitative PCR quantifies transcription of a specific gene by detecting the release of a fluorescent reporter dye. Forward and reverse primers recognize the target DNA sequence, whilst a probe recognizes a sequence between the annealing sequences of the two primers. This probe is labeled with two fluorescent dyes: a 5' reporter dye (see below) and a 3' quencher dye (TAMRA; 6-carboxytetramethylrhodamine). When the intact probe is annealed to the target DNA the quencher dye is in close proximity to the reporter dye, and suppresses its fluorescence. When the target sequence is amplified during a PCR reaction the probe is cleaved by the endonuclease activity of Taq polymerase, resulting in the separation of the two dyes so that the quencher dye can no longer suppress the reporter fluorescence (Figure 2.2). Fluorescence is thus increased allowing measurement of PCR product. Detection of non-specific amplification is avoided because fluorescence is detected only if the probe's target sequence is amplified.

The amount of ribosomal 18S in samples is constant, relative to the amount of cDNA present. Concurrent measurement of ribosomal 18S and a specific amplicon is possible through labelling with reporter dyes emitting different wavelengths (18S uses VIC; chemical name not released: all other amplicons use FAM; 6-carboxyfluorescein). Quantification of the specific amplicon can be thus related to the abundance of ribosomal 18S. Subsequent analysis relates this amount to the amount in an internal control (see below).

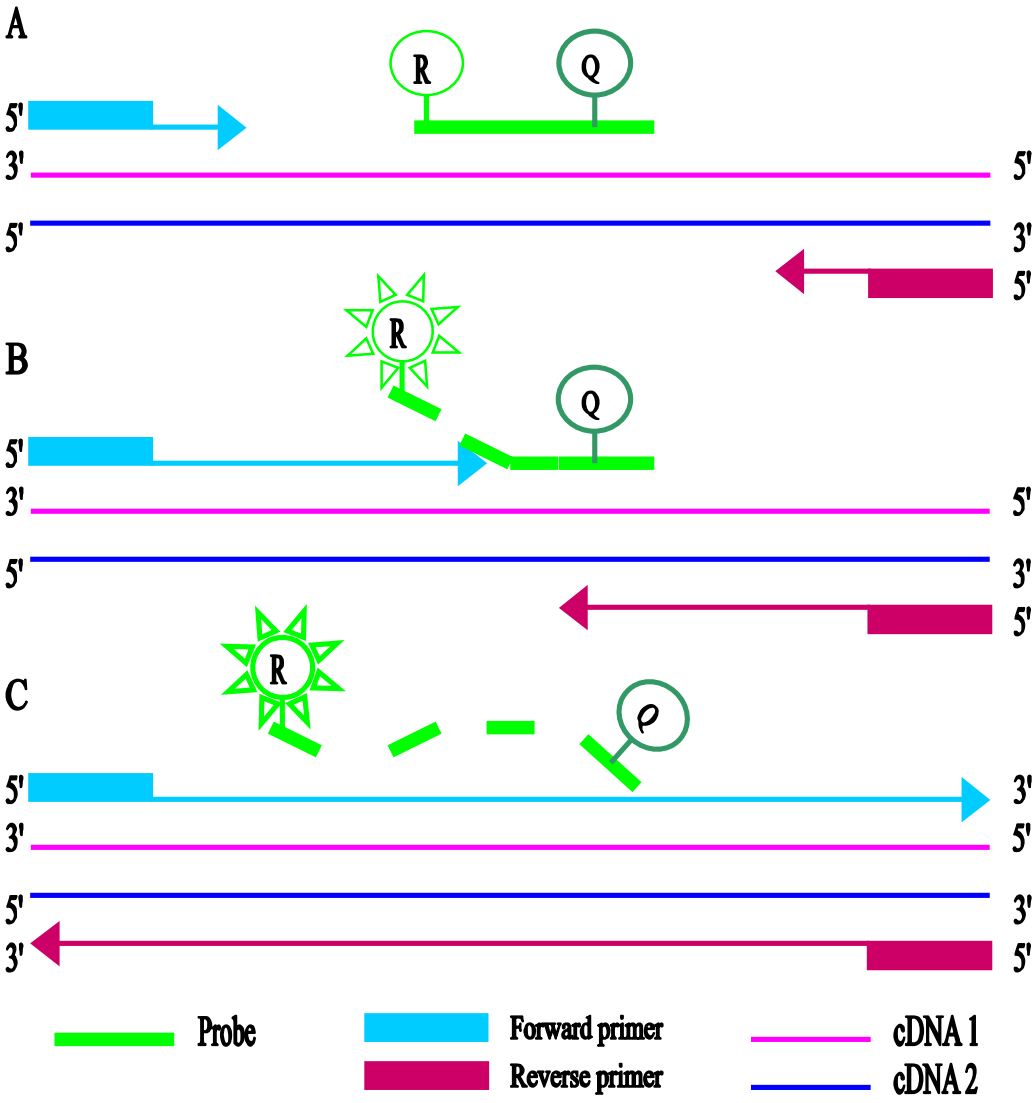


Figure 2.2

Taqman quantitative PCR Reaction.

A. Polymerization: Primers and probe polymerize to template cDNA. Fluorescence is low as the quencher dye (Q) suppresses the fluorescence of the reporter dye (R).

B. Displacement and cleavage: As the primer extends along the template, the probe is displaced and then cleaved by the actions of Taq polymerase. This separates the reporter dye from the suppressive effects of the quencher.

C. Polymerization is completed: The fluorescence increases and this allows measurement of the amount of PCR product generated.

(Adapted from the Taqman PCR Reagent Kit Protocol.)

2.4.1. Method

A reaction mix was made from Stratgene PCR mastermix, 18S control primers and probes (50nM), and forward and reverse primers (300nM) and hybridization probe (200nM probe) specific to the gene of interest. Samples were run in at least duplicate, in Taqman Fast optical PCR plates (Applied Biosystems), with each replicate comprising of 2µl of cDNA and 18µl reaction mix. Three negative controls were included on each PCR plate. An RT-negative sample generated at the time of reverse transcription (RNA template with no reverse transcriptase enzyme) was used to exclude genomic DNA contamination. An RT-H_2O sample generated at the time of reverse transcription (water in place of RNA template) was used to ensure specificity of reversed transcription. A TaqMan-reaction negative control where cDNA was replaced with water was also included. Wells were sealed with optical cover (Applied Biosystems) and the PCR reaction run on ABI Prism 7900.

2.4.2. Analysis

The amount of target mRNA was normalized to the amount of 18S RNA and this value was related to that of an internal control, using the formula $2^{-\Delta\Delta CT}$. CT is the cycle number at which the PCR signal crosses a threshold. ΔCT is the difference between the CT values for the specific amplicon and 18S. ΔΔCT refers to the difference between the ΔCT value of the sample and the internal control. Thus $2^{-\Delta\Delta CT}$ gives a value showing the fold difference in the amount of amplicon compared to control.

2.4.3. Primer and probes

Primers and probes were designed using the PRIMER express program (Perkin-Elmer Biosystems, MA, USA). In an effort to avoid amplification of any contaminating genomic DNA, probes were designed to span an intron junction between two exons. If this was not possible then primers were taken from separate exons. The sequences for the primers and probes used are shown in Table 2.1.

BLAST (Basic Local Alignment Tool) searches (www.ncbi.nlm.nih.gov) were performed to determine the presence of sequences in the scientific databases that are similar to those amplified by each set of primers. The results from these include an expect value (E) which indicates the number of sequences which similarly match the sequence of interest, that could be expected to be found by chance when searching the databases. An E value of 1 would suggest that 1 sequence with similar matching could be found by chance. The results of these searches showed that, in all cases, the primer and probe sets used were unlikely to amplify an inappropriate template. E values for each primer probe set are shown in Table 2.1.

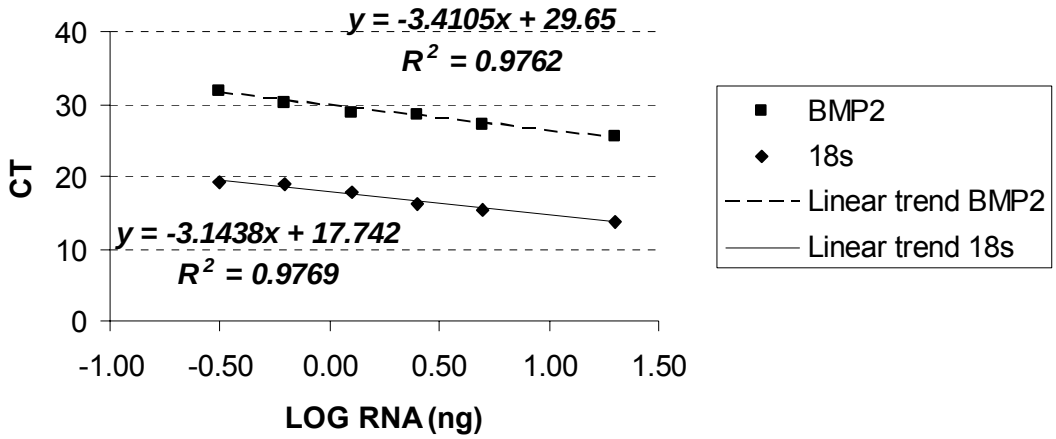
Primers and probes were validated using serially diluted RNA. Analysis of PCR data by the ΔΔCT method is dependant on 18S and the gene of interest replicating with similar efficiencies. This was determined by plotting the CT value against the logarithm of the total amount of RNA in ng. The slopes of the lines should approximate -3.3 and have a correlation co-efficient of >0.95. Representative data is shown in Figure 2.3. Within assay variation of the PCR measurement of specific amplicon in cDNA was calculated from 6 replicates (relative standard deviation), and accepted if <10%.

Name	Forward Primer	Reverse Primer	Probe	E
BMP2	CATGATGGAAAAGGGCATCT	AGGCGTTTCCGCTGTTTG	TCCACAAAAGAGAAAAACGTCAAGCCAAAC	8.00E-07
CD14	GCGCTCCGAGATGCATGT	AGCCCAGCGAACGACAGA	TCCAGCGCCCTGAACTCCCTCA	4.00E-02
CD69	CCATTTCTCAACACGTCATGG	TGGCCCACTGATAAGGCAAT	TCCTTCCAAGTTCCTGTCCTGTGTGCTG	1.00E-05
COX2	GTGTTGACATCCAGATCACATTTGA	GAGAAGGCTTCCCAGCTTTTGTA	TGACAGTCCACCAACTTACAATGCTGACTATGG	1.00E-05
CXCL8	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG	CCTTCCTGATTTCTGCAGCTCTGTGTGAA	2.00E-08
Elafin	TGGCTCCTGCCCCATTATC	CAGTATCTTTCAAGCAGCGGTTAG	ATCCGGTGCGCCATGTTGAATCC	3.00E-03
HBD1	TCAGCAGTGGAGGGCAATG	CCTCTGTAACAGGTGCCTTGAAT	CTCTATTCTGCCTGCCCGATCTTTACCAA	8.00E-07
HBD2	CTGATGCCTCTTCCAGGTGTTT	CTGGATGACATATGGCTCCACTCT	AAGGCAGGTAACAGGATCGCCTATACCACCA	2.00E-17
HBD3	CAGAGGCGGCCGGTGT	CGAGCACTTGCCGATCTGTT	CTGTGCTCAGCTGCCTTCCAAAGGA	6.00E-04
HBD4	GGCAGTCCCATAACCACATATTC	TGCTGCTATTAGCCGTTTCTCTT	TGTCCAATTCAAATTCGCTTCTCACTGGA	4.00E-06
IL-1α	TGTATGTGACTGCCCAAGATGAA	CTACCTGTGATGGTTTTGGGTATC	CAACCAGTGCTGCTGAAGGAGATGCCTG	4.00E-06
IL-1β	CGGCATCCAGCTACGAATCT	CATGGCCACAACAACTGACG	CGACCACCACTACAGCAAGGGCTTCAG	5.00E-05
IL-1RA	TTGCAAGGACCAAATGTCAATT	CCATGGATTCCCAAGAACAGA	CATGAGGCTCAATGGGTACCACATCTATCTTTT	2.00E-17
NFκBIα	TTGGGTGCTGATGTCAATGC	AGGTCCACTGCGAGGTGAAG	AGGAGCCCTGTAATGGCCGGACTG	3.00E-03
NFκBIβ	CAGCATGAACCCTTCCTGGAT	GTCATTCTGCAGGTCCATGTACTC	TCTTCTAGGCTTCTCGGCCGGCAC	3.00E-03
NFκBIζ	GGAAGCGAAGGATCGCAAA	CATTCACAAAAGACAGGCAACTG	CTGGAACTCATTCGCCTCTTTTTGGAGC	1.00E-05
SLPI	GCATCAAATGCCTGGATCCT	GCATCAAACATTGGCCATAAGTC	TGACACCCCAAACCCAACAAGGAGG	8.00E-0.4
TLR2	CTGCAAGCTGCGGAAGATAAT	GCAGCTCTCAGATTTACCCAAAAT	AAGACCTACCTGGAGTGGCCCATGGAC	5.00E-05
TLR4	CTGCCACATGTCAGGCCTTA	TTCCCTCTGCACTGGAAGCT	CTAAGGGTGAGTAATTCCATGGTGCACTAGATATGC	2.00E-10
TNFα	GGAGAAGGGTGACCGACTCA	TGCCCAGACTCGGCAAAG	CGCTGAGATCAATCGGCCCGACTA	2.00E-04

Table 2.1

Sequences of primer probe sets used for Taqman quantitative PCR, with BLAST expect value (E). The lower the E value, the lower the chance that another sequence matches the sequence of interest.

A



B

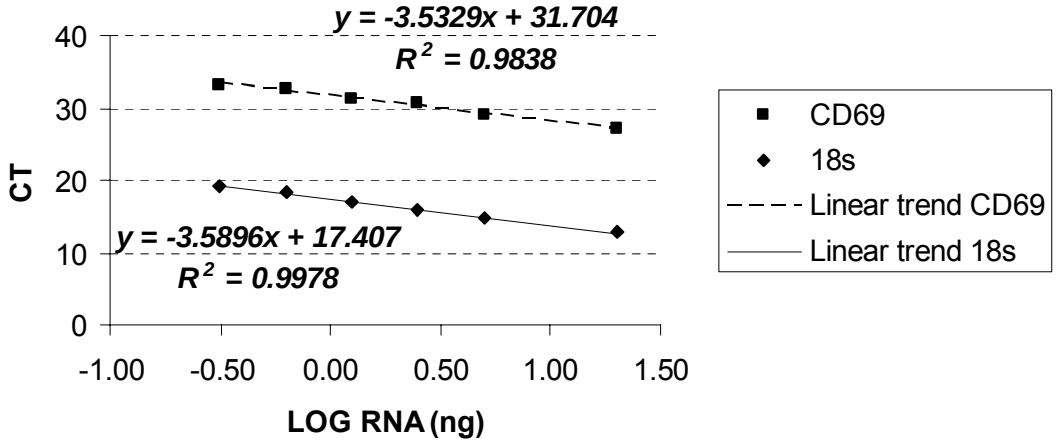


Figure 2.3

Validation of BMP2 (A) and CD69 (B) primer probe sets.

The CT (cycle threshold) value is plotted against the logarithm of the total amount of RNA in ng. If the gene of interest and 18s primer and probes set amplify with equal efficiency the slopes of the lines should approximate -3.3 and have a correlation co-efficient of >0.95. Intra-assay variation was 4.9% and 4.11% respectively. Other primer probe sets gave similar results.

2.5. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs can quantify the amount of a given protein in a solution, through comparison with a standard curve created from samples containing known concentrations. The assays performed in this project were all two-site ELISAs.

A two-site or sandwich ELISA uses plates coated with an antibody raised against one epitope of the substance to be measured. The solution of interest, or the standard, is then added, and the substance binds to the antibody. Next a detection antibody is added, which is raised against a second epitope and labelled with biotin. Streptavidin peroxidase is then added. Streptavidin complexes strongly with the biotin and the peroxidase can be detected by addition of substrate (Tetramethyl benzidine, urea hydrogen peroxide, sodium acetate; Appendix 2). The reaction is stopped with sulphuric acid solution and absorbance measured by spectrophotometry OD_{450} (Figure 2.4).

Standard curves were constructed and sample measurements calculated by the Assay Zap program (Assay Aqp, BioSoft, Cambridge UK). Assays were validated by performing serial dilutions of a sample and confirming that when the absorbance was plotted a linear response was obtained which was parallel to the standard curve. Representative data is shown in Figure 2.5. Assay precisions were calculated using the formula “standard deviation/mean x 100” to give the relative standard deviation (rsd). The intra-assay coefficient of variation (rsd) was determined from at least 6 replicates of a quality control sample. The inter-assay coefficient of variation (rsd) was determined from the quality control values of at least 6 different plates. However, this was not calculated for all ELISAs because in some experiments all values were obtained from one assay.

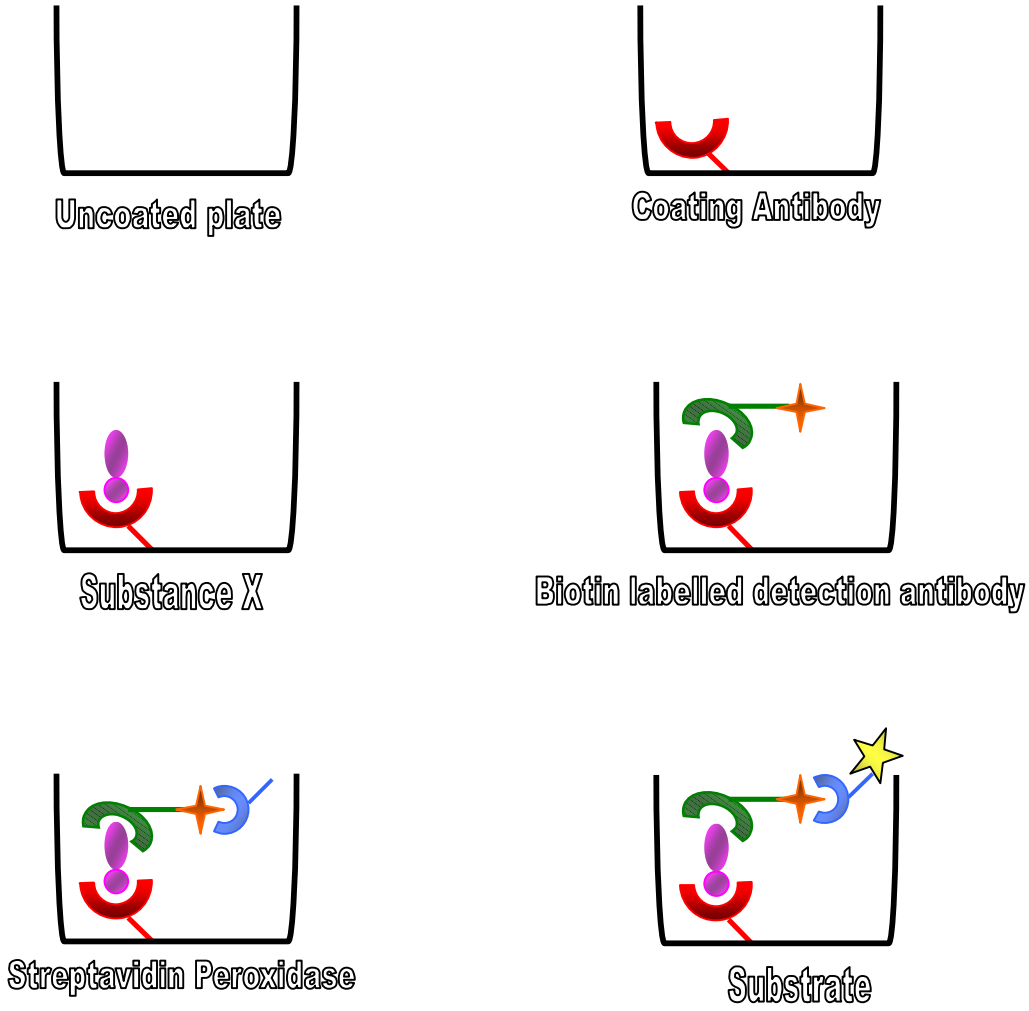
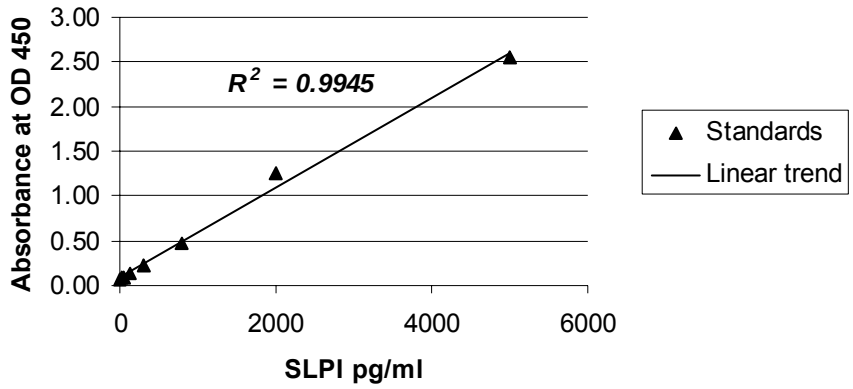


Figure 2.4

Sandwich ELISA

Plates are coated with an antibody raised against substance to be measured. When the solution of interest, or the standard, is added the substance binds to the antibody. A biotin labelled detection antibody is then applied which binds to substance X using a different epitope. Streptavidin peroxidase complexes with the biotin and the addition of substrate produces a differential colour shift, measurable by spectrophotometry.

A



B

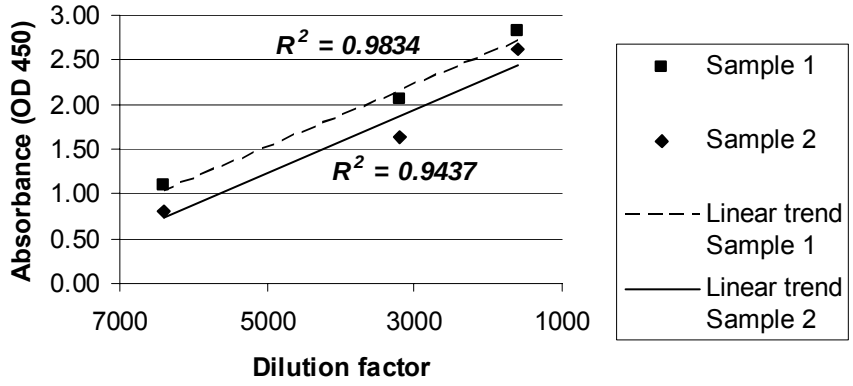


Figure 2.5

Validation of SLPI ELISA.

Absorbance at OD 450nm of SLPI standards in pg/ml (A) or the dilution factor of serially diluted samples (B), showing parallel linear responses. Other assays gave similar results.

2.5.1. HBD2 ELISA

An assay development kit containing capture antibody, biotinylated detection antibody, and recombinant HBD2 was used. 96-well plates were coated with goat polyclonal antiHBD2 (100µl/well, 25µg/ml) diluted in PBS, and incubated at room temperature overnight. Non-specific binding was blocked by the addition of 200µl per well of dry coat and a further incubation for 2 hours at room temperature. Plates were washed four times with wash buffer. Standards of recombinant human HBD2 were serially diluted in ELISA buffer with tween, from 1000pg/ml to 1.95pg/ml. 100µl of standard or sample were applied in duplicate, and incubated at 4°C overnight. The following day plates were washed four times, before application of biotin labelled detection antibody diluted in ELISA buffer with tween (100µl/well, 5µg/ml). Plates were incubated for 2 hours on an orbital plate shaker, before being washed another four times. 100µl per well of streptavidin peroxidase at 0.02IU was added, and incubated for 15 minutes, again on an orbital plate shaker. Plates were thoroughly washed six times, and 200µl per well of substrate added. Colour was allowed to develop for approximately 5 minutes, at which point 50µl per well of 2N sulphuric acid was added to stop the reaction. Plates were read at an optical density of 450nm within 30 minutes of quenching. The intra-assay precision was 8% rsd, the inter-assay precision was 10% rsd and the detection limit of the assay was 8pg/ml.

2.5.2. HBD3 ELISA

HBD3 assay was performed in a similar way to the HBD2 assay, using reagents from an assay development kit. Capture antibody was applied at a concentration of 3µg/ml, standards ranged from 4000 to 7.8pg, and detection antibody was used at a concentration of 0.25µg/ml. The intra-assay precision was 4.9 % rsd and the detection limit of the assay was 62 pg/ml.

2.5.3. IL-8 ELISA

IL-8 ELISA was performed in a similar way to the HBD2 assay, using matched pairs of capture antibody and biotinylated detection antibody, and recombinant human IL-8. Capture antibody was applied at a concentration of 2µg/ml, standards ranged from 1000 to 7.8pg/ml and detection antibody was used at a concentration of 62.5ng/ml. The intra-assay precision was 9.4% rsd, the inter-assay precision was 7.7% rsd and the detection limit of the assay was 7.8pg/ml.

2.5.4. IL-1β ELISA

IL-1β ELISA was performed in a similar way to the HBD2 assay, using matched pairs of capture antibody and biotinylated detection antibody, and recombinant human IL-1β. Capture antibody was applied at a concentration of 2µg/ml, standards ranged from 10,000 to 78pg/ml and detection antibody was used at a concentration of 62.5ng/ml. The intra-assay precision was 3% rsd, the inter-assay precision was 23.2% rsd and the detection limit of the assay was 78pg/ml.

2.5.5. IL-1RA ELISA

IL-1RA ELISA was performed in a similar way to the HBD2 assay, using matched pairs of capture antibody and biotinylated detection antibody, and recombinant human IL-1RA. Capture antibody was applied at a concentration of 5µg/ml, standards ranged from 10,000 to 78pg/ml, and detection antibody was used at a concentration of 83ng/ml. The intra-assay precision was 5.2% rsd, the inter-assay precision was 9.8% rsd and the detection limit of the assay was 78pg/ml.

2.5.6. TNFα ELISA

TNFα ELISA was performed in a similar way to the HBD2 assay, using matched pairs of capture antibody and biotinylated detection antibody, and recombinant human TNFα. Capture antibody was applied at a concentration of 2µg/ml, standards

ranged from 2000 to 31.2pg/ml, and detection antibody was used at a concentration of 100ng/ml. The intra-assay precision was 3.8% rsd, the inter-assay precision was 5.0% rsd and the detection limit of the assay was 31.2pg/ml.

2.5.7. SLPI ELISA

A SLPI assay kit was used, containing plates precoated with capture antibody, and all required reagents and buffers. Samples and standards (100µl, range 5000 to 20pg/ml) were diluted in dilutant provided, applied and incubated at 37°C for 1 hour. Plates were washed with wash buffer, and detection antibody (100µl) added. Plates were incubated for 2 hours on an orbital plate shaker at room temperature, and then washed again. Streptavidin peroxidase was applied to each well (100µl, 0.02IU) and incubated for 15 minutes. 100µl of the provided substrate was then added after a further wash, and the reaction was quenched with stop solution (100µl). The intra-assay precision was 7.8% rsd; the inter-assay precision was 6.5% rsd and the detection limit of the assay was 20pg/ml.

2.5.8. ELAFIN ELISA

A kit was also used for the elafin assay, containing plates precoated with capture antibody, and all required reagents and buffers. Samples and standards (100µl, range 10,000 to 156pg/ml) were applied and incubated at room temperature for 2 hours. Plates were washed with wash buffer, and detection antibody (100µl) added. Plates were incubated for 2 hours on an orbital plate shaker at room temperature, and then washed again. Streptavidin peroxidase was applied to each well (100µl, 0.02IU) and incubated for 15 minutes. 100µl of the provided substrate was then added after a further wash, and the reaction was quenched with stop solution (100µl). The intra-assay precision was 10.8% rsd, the inter-assay precision was 3.1% rsd and the detection limit of the assay was 160pg/ml.

2.5.9. Total Protein Assay

To avoid bias secondary to variation in cell numbers, the amount of total cellular protein was determined whenever media from primary cells was analysed by ELISA and used as a denominator in analysis of results. After media was harvested and stored at -20°, cultured cells were washed twice with cold PBS. 300µl of protein lysis buffer, supplemented with protease inhibitor, was added to each well, and incubated on ice for 10 minutes. Cells were detached using a cell scraper, and incubated for another 10 minutes. Lysate was repetitively drawn through a pipette tip ten times, and then centrifuged at 8000g for 15 minutes at 4°C. Supernatant was stored at -20°C until further use.

The amount of protein in cultured cells and cervicovaginal secretion samples was measured using a Biorad commercial assay. This contains an acidic dye that binds to protein and exhibits a differential colour shift detectable by spectrophotometry. Comparison with a standard curve provides a measurement of protein concentration.

Standard curves were generated using bovine serum albumin (BSA) diluted in distilled water to concentrations from 2.29µg/ml to 294µg/ml. 25µl of standard or appropriately diluted sample were added to each well of a 96 well plate in at least duplicate. 5ml of reagent A was added to 100µl of reagent S and 25µl of the mixture was added to each well. 100µl of reagent B was then added to each well and colour development monitored. Plates were read in an OD spectrophotometer in absorbance mode, wavelength 690nm after 10 minutes.

2.6. IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY

2.6.1. Haematoxylin and Eosin (H and E)

H and E staining was used to check morphology of tissue samples. 5µ tissue sections were dewaxed in xylene for 5 minutes, rehydrated in descending grades of ethanol, and washed in H_2O. They were stained in haematoxylin for 2 minutes, then rinsed in

1% acid alcohol (1-5 seconds) and Scott's tap water (30-60seconds) with washes in H_2O in between. Slides were then counterstained with Eosin (5-10 seconds), rinsed, and rehydrated in ascending grades of alcohol. They were mounted from xylene with pertex mounting medium.

2.6.2. Cytokeratin

The amnion is comprised of a continuous monolayer of epithelial cells, with a more sparsely distributed fibroblast layer underneath. An antibody for pancytokeratin was used to identify the epithelial layer in tissue sections and confirm the epithelial origin of cultured primary amnion cells. Cultured cells were fixed in neutral buffered formalin (NBF) for 30 minutes at 23°C, then stored in ethanol 70 % at 4°C. Cells and tissue sections (5µ) were rehydrated in descending grades of ethanol. Cells were permeabilised in 0.05% Triton for 10 minutes, whereas tissue sections underwent antigen retrieval by pressure cooking in sodium citrate for 5 minutes. After washing in PBS, samples were incubated with methanol H_2O_2 to inhibit endogenous peroxidase activity which can cause non-specific staining. Blocking serum was added for 30 minutes to block non-specific binding. Mouse antibody to pancytokeratin (1:100 in blocking serum) was then added and samples were incubated in a humidified chamber overnight at 4 °C. A no antibody control was included in each run. The next day the cells/slides were washed twice in PBS with Tween (PBST), and a secondary antibody (rabbit anti-mouse, 1:500 in blocking serum) added for 60 minutes. A further 2 washes with PBS were performed, and then Avidin-Biotin Complex (ABC) applied for 30 minutes. After a further 2 washes with PBST, 3, 3 –diaminobenzidine (DAB) was added to produce a brown stain. Harris's haematoxylin was applied as a counterstain for 2 minutes. Cells were stored in distilled water, and tissue sections were rehydrated in ascending grades of alcohol and mounted from xylene with pertex mounting medium.

2.7. STATISTICAL ANALYSIS

Analysis was carried out with the software package Graphpad Prism. Statistical tests used are described in each chapter. $P<0.05$ was regarded as significant.

3. Natural Antimicrobial Production by the Cervix and Vagina

3.1. INTRODUCTION

The lower genital tract is constantly exposed to pathogenic bacteria, yet infections occur comparatively rarely. It is well recognised that the epithelia form a physical barrier to infection, and the resident lactobacilli create an inhospitable environment for pathogens. However, there is a growing body of evidence suggesting the vaginal and cervical epithelia also actively contribute to microbial elimination via their innate immune response. This chapter investigates the innate immune response of the cervix and vagina, and its relationship to the commonest infection of the lower genital tract – bacterial vaginosis.

Bacterial vaginosis is characterized by a deficit of the lactobacilli that normally populate the vagina, and overgrowth of mixed anaerobic bacteria. It does not cause a classical inflammatory reaction and in the majority of cases it is asymptomatic. Nonetheless, it is associated with increased incidence of a number of infective pathologies, in particular preterm labour, preterm rupture of membranes and late miscarriage (Guaschino, De Seta et al. 2006).

The hypothesis that bacterial vaginosis is associated with deranged levels of natural antimicrobials and cytokines is examined in this section. Cervicovaginal secretions were collected from pregnant women with normal flora, intermediate flora, and bacterial vaginosis, and differences in antimicrobial and cytokine concentrations were measured. In addition, vaginal, endocervical and ectocervical epithelial cell lines were used to study *in vitro* production of natural antimicrobials, and to compare the characteristics of the innate immune response in the different tissue types.

3.2. METHODS

All materials, reagents and cell lines used are detailed in Appendix 1.

3.2.1. Specimen Collection

Cervicovaginal secretions were obtained from women with uncomplicated singleton pregnancies attending community antenatal clinics. Women were excluded if they had placenta praevia, vaginal bleeding, known current urinary tract or sexually transmitted infection or reported antibiotic use within the previous two weeks.

Samples were self-collected by applicator tampon insertion for 15 minutes and removed to 10ml of PBS. This method of collection is relatively non-invasive and has been shown to be acceptable to women, safe and effective (Wilkinson, Ndovela et al. 1997). Duplicate slides were immediately made from samples using a method adapted from Sturm et al (2002), shown to have excellent agreement for diagnosis of bacterial vaginosis when compared with vaginal smear (Sturm, Moodley et al. 2002). PBS was drawn up and re-expressed through the tampon three times using a sterile 20 ml. A drop of the eluted fluid was then allowed to air dry on a microscope slide, for subsequent Gram stain. The tampon was replaced in the remaining PBS, and transported to the laboratory where they were stored at 4°C and processed within 24 hours of collection.

Preliminary experiments were carried out on 43 samples obtained from women at any gestation to validate the method. Levels of SLPI were found to be significantly greater in samples from women who reported having sexual intercourse in the previous 24 hours than in those who denied having intercourse. It was assumed that this was secondary to high levels of SLPI in seminal fluid, and in subsequent experiments women were excluded if they gave a history of sex in the 48 hours prior to sampling. A history of recent bathing had no significant effect on natural antimicrobial levels. Results from a simple questionnaire given to the women indicated that the test was acceptable.

Subsequent experiments were carried out on samples obtained only from women under 20 weeks gestation. 115 samples were collected in total. Two samples were excluded, as slides for vaginal flora assessment were inadequate or lost.

3.2.1.1. *Extraction of secretions*

10mls of acetic acid 0.25% was added to the tube containing the tampon and remaining PBS, and the solution was expressed and redrawn through the tampon six times to ensure release of basic proteins and peptides. Preliminary experiments compared the weight of a tampon prewetted with 1ml of fluid (to mimic vaginal fluid volume) before and after elution with acetic acid. This showed over 99% of the sample was excreted from the tampon using this method (n=10). The eluted fluid was centrifuged at 380 G for 15 minutes to remove debris, divided into 5ml aliquots, lyophilized and stored at -80°C.

3.2.1.2. *Gram stain*

Slides of cervicovaginal secretions were flooded with crystal violet oxalate for 2 minutes and iodine for 3 minutes, then washed with acetone and counterstained with neutral red for 3 minutes. They were air dried, and mounted with pertex mounting medium.

3.2.2. Diagnosis of Bacterial Vaginosis

Diagnosis of bacterial vaginosis was made using Nugent's criteria (Nugent, Krohn et al. 1991). Four x1000 oil immersion fields per slide were viewed and the average number of *Lactobacilli, Gardnerella vaginalis spp/Bacteroides spp*, and Gram variable rods per field determined. A total score of 0-10 was calculated using Nugent's scoring system (Table 3.1). A score of 0-3 was considered normal, whereas 4-6 indicated an intermediate floral pattern and 7-10 diagnosed bacterial vaginosis.

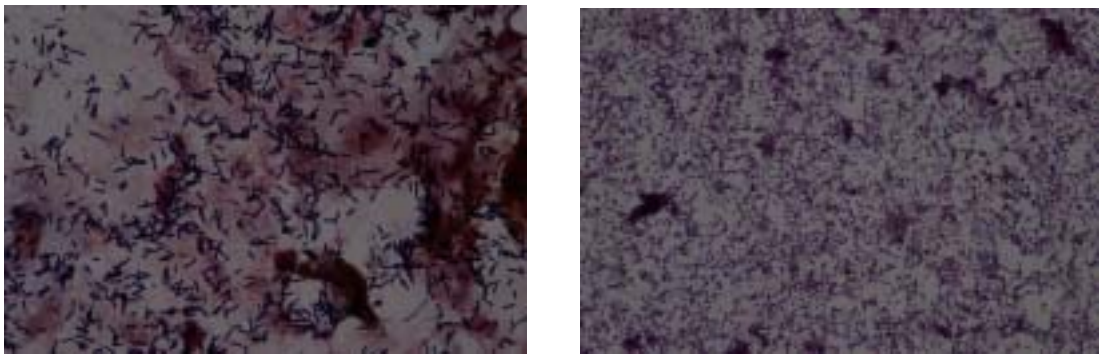
Score[B]	*Lactobacillus Morphotypes*	*Gardnerella and Bacteroides*	*Curved Gram Variable Rods*
0	4+	0	*0*
1	3+	1+	*1+ or 2+*
2	2+	2+	*3+ or 4+*
3	1+	3+	
4	0+	4+	

Table 3.1

Nugent's scoring system (0-10) for Gram-stained vaginal smears [a].

[a]Morphotypes are scored as the average number seen per oil immersion field. Total score = *Lactobacilli* + *Gardnerella vaginalis* and *Bacteroides spp* + curved rods.

[b]0= No morphotypes present; 1 < 1 morphotype present; 2 = 1-4 morphotypes present; 3= 4-30 morphotypes present; 4= >30 morphotypes present.



A **B**

Figure 3.1

Gram stained smears of cervicovaginal secretions.

Figure A shows normal bacterial flora, where *lactobacilli* predominate. Figure B shows bacterial vaginosis, characterized by a paucity of *lactobacilli* and overgrowth of Gram poitive and Gram negative organisms.

3.2.3. Total Protein Assay

The amount of protein in cervicovaginal secretion samples was measured as detailed in Section 2.5.9.

3.2.4. ELISA

Levels of HBD2, HBD3, ELAFIN, SLPI, IL-8, IL-1β and IL-1RA were measured in cervicovaginal secretion samples by ELISA, as described in Section 2.5.1-8.

3.2.5. VK2 E6/E7, END E6/E7 and ECT E6/E7 cell culture

VK2 E6/E7, END E6/E7 and ECT E6/E7 cells were cultured as described in section 2.2.1.3. Cells were treated with lipopolysaccharide (LPS; 1μg/ml), lipoteichoic acid (LTA; 500ng/ml), recombinant human IL-1β (10ng/ml) or progesterone (1μM) or equivalent volumes of vehicle (serum free media for bacterial wall products and cytokines; ethanol for progesterone) for 0 or 24 hours. Treatments were performed in quadruplicate. RNA was extracted from one set of duplicates for analysis of natural antimicrobial and cytokine expression by Taqman quantitative PCR (method and primer and probes detailed in Section 2.3-4). A pooled sample from END E6/E7 cells was used as a positive control. Media was harvested from the other set of duplicates, stored at -20°C and analyzed by ELISA (Section 2.5).

3.2.6. Statistical analysis

Categorical data was analyzed using chi-square test, with confidence intervals determined by the modified Wald method. Continuous data was analyzed using the Kolmogorov-Smirnov test for normality. Parametric data was analyzed using paired t-test (2 groups) or one-way ANOVA (3 or more groups) with Tukey's test to assign individual differences. Non-parametric data was analyzed using the Kruskal-Wallis test. Graphs of parametric data represent mean +/- SEM. Graphs of non-parametric data represent median +/- interquartile range (box) and range (whiskers). $P<0.05$ was regarded as significant.

3.3. RESULTS

3.3.1. Sample characteristics

58.1% (65/112) of women sampled had normal flora, 17.8% (20/112) had intermediate flora and 24.1% (27/112) had bacterial vaginosis. There were no significant differences between the groups in terms of age, gestation at sampling, or ethnic origin. The group with bacterial vaginosis contained more smokers than the other groups (Table 3.2).

	Normal Flora	*Intermediate flora*	*Bacterial Vaginosis*	*P value*
n	***65***	***20***	***27***	-
Age[a]	31.9 (0.5)	31.6 (1.2)	30.2 (1.0)	*0.3*
Gestation[a]	13.7 (0.2)	13.8 (0.4)	13.7 (0.3)	*0.9*
Caucasian[b]	93.8% (84.8 –98.0)	100% (81.0-100)	96.3% (80.2-99.9)	*0.4*
Non smokers[b]	95.3% (86.6-98.9)	90.0% (68.7-98.4)	74.1% (55.1-87.1)	***0.03***

Table 3.2

Characteristics of women from whom cervicovaginal secretion samples were obtained. [a]Data expressed as mean (+/- SEM) and analyzed by one-way ANOVA. [b]Data expressed as percentage (95% confidence interval) and analyzed by chi-square test.

3.3.2. Follow-up data

This study was not powered for correlation of outcome with either bacterial vaginosis status or natural antimicrobial levels. However, delivery details were collected where possible, to enable use in any future larger trials.

Data was available for 100 of the women involved in the study. Data was missing from five women who had moved care to a different maternity unit after booking. It was not possible to establish reasons for the other cases of loss to follow up, but fetal loss before 24 weeks is a possible reason why this information was not available on the obstetric data management system.

Two women had stillbirths, one at 28 weeks and one at 35 weeks. There were nine further deliveries before 37 weeks (preterm delivery rate of 9.2%). Seven of these occurred before 34 weeks. Of the women who delivered preterm, 6 had a diagnosis of bacterial vaginosis (66.7%), 1 had intermediate flora, and 2 had normal flora.

3.3.3. Bacterial vaginosis status and protein levels in cervicovaginal secretions

The total protein concentration was lower in cervicovaginal secretion samples from women with bacterial vaginosis (median 28.7 mg/ml; interquartile range 23.1-39.1) than from those with normal (median 41.9 mg/ml; interquartile range 34.9-50.8) or intermediate flora (median 39.3 mg/ml; interquartile range 28.7-48.8) (Figure 3.2; $P<0.001$).

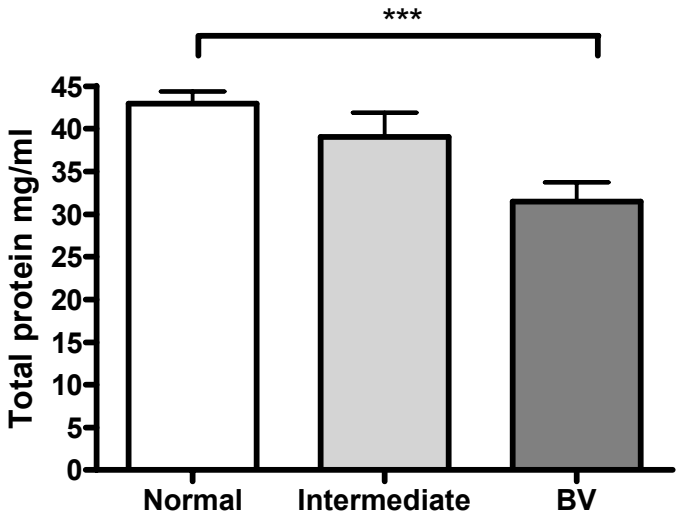


Figure 3.2

Mean (mg/ml) +/- SEM concentration of total protein in cervicovaginal secretions obtained from women in the second trimester of pregnancy with normal vaginal flora, intermediate vaginal flora, or bacterial vaginosis. ***P<0.001 determined by one-way ANOVA with Tukey's post-test.

3.3.4. Bacterial vaginosis status and levels of natural antimicrobial in cervicovaginal secretions

Cervicovaginal secretions were analysed for the natural antimicrobials HBD2, HBD3, SLPI and elafin using ELISA.

Elafin was found in all 112 samples of cervicovaginal secretions, at much higher concentrations than the defensins (range 987.3-10000ng/ml). The median concentration of elafin in samples obtained from women with bacterial vaginosis (1343 ng/ml; interquartile range 1150-1547) was significantly lower than those of samples obtained from women with normal (1608 ng/ml; interquartile range 1319-2319) ($P<0.01$) or intermediate flora (1543 ng/ml; interquartile range 1341-2080) ($P<0.05$; Figure 3.3).

SLPI was also detectable in all 112 samples of cervicovaginal secretions (range 24.5 – 12451 ng/ml). The median concentration of SLPI in samples obtained from women with bacterial vaginosis (1344 ng/ml; interquartile range 588-2051) was significantly lower than that of samples obtained from women with normal flora (2240; interquartile range 997-4108) ($P<0.05$; Figure 3.4).

HBD2 was found in 110 out of 112 samples (range 0-2306 pg/ml). There was no difference in median HBD2 concentrations in samples from women with normal flora (median 663 pg/ml; interquartile range 340-2084), intermediate flora (median 1205 pg/ml; interquartile range 430-2270), or bacterial vaginosis (median 632 pg/ml; interquartile range 192-2677). ($P=0.61$; Kruskal-Wallis test).

HBD3 was the least abundant natural antimicrobial; being detectable in only 31 out of 112 (27.6%) samples of cervicovaginal secretions (range 0-1998 pg/ml). There was no significant difference in the proportion of HBD3 negative samples in women with normal flora (73.8%), intermediate flora (65%) and bacterial vaginosis (74.1%). ($P=0.65$; chi-square).

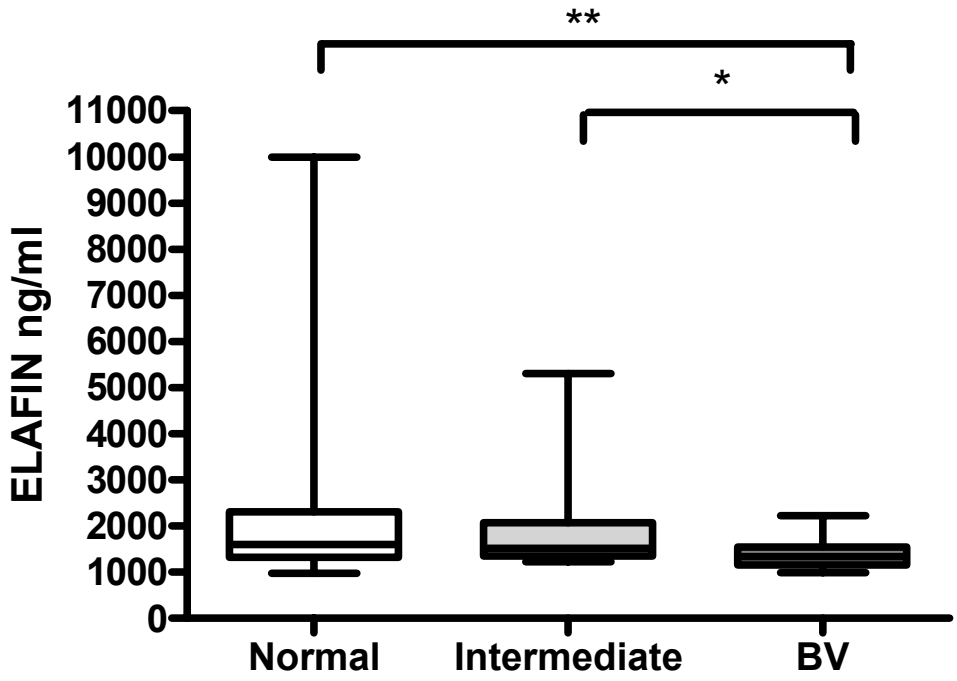


Figure 3.3

Median concentrations of Elafin (ng/ml) +/- interquartile range (box) and range (whiskers) in cervicovaginal secretions obtained from women in the second trimester of pregnancy with normal vaginal flora (n=66), intermediate vaginal flora (n=20), or bacterial vaginosis (n=27). *P<0.05 and **P<0.01 determined by Kruskal-Wallis test.

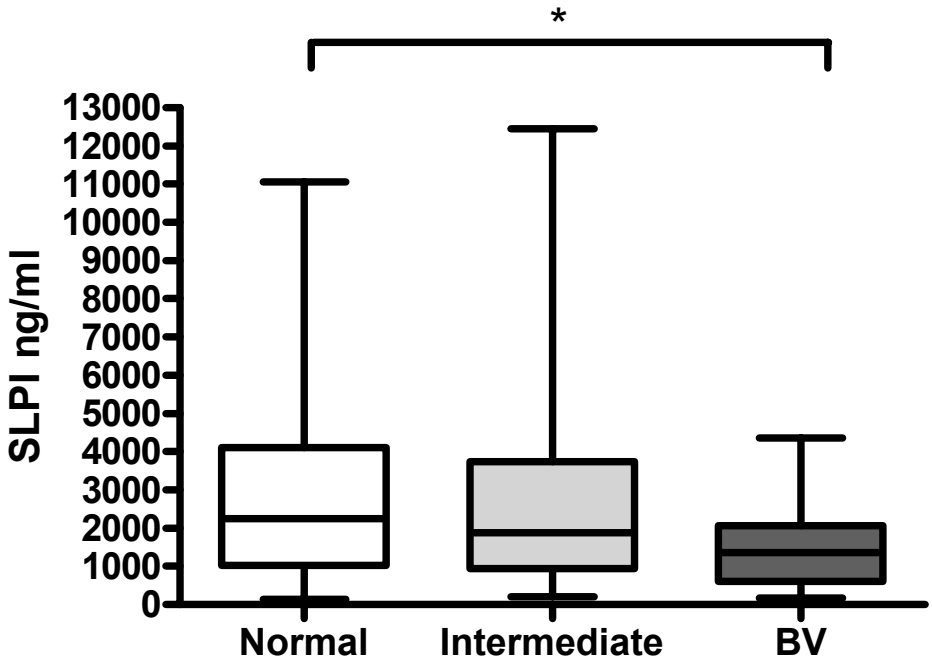


Figure 3.4

Median concentrations of SLPI (ng/ml) +/- interquartile range (box) and range (whiskers) in cervicovaginal secretions obtained from women in the second trimester of pregnancy with normal vaginal flora (n=66), intermediate vaginal flora (n=20), or bacterial vaginosis (n=27). *$P<0.05$ determined by Kruskal-Wallis test.

3.3.5. Bacterial vaginosis status and levels of cytokines in cervicovaginal secretions

Cervicovaginal secretions were analysed for IL-1β, IL1RA, IL-8, and CCL5/RANTES using ELISA. IL-1β and IL-8 are proinflammatory cytokines which can stimulate production of natural antimicrobials. Along with IL1RA, they have been examined in other studies of bacterial vaginosis (Imseis, Greig et al. 1997; Mattsby-Baltzer, Platz-Christensen et al. 1998; Wennerholm, Holm et al. 1998; Spandorfer, Neuer et al. 2001; Cauci, Guaschino et al. 2003; Basso, Gimenez et al. 2005; Diaz-Cueto, Cuica-Flores et al. 2005; Hedges, Barrientes et al. 2006). Comparison of these results could thus be used as validation of the sample collection technique. CCL5/RANTES is another chemokine which has been reported to be secreted by cervical cells (Fichorova and Anderson 1999) but has not been examined in cervicovaginal secretions to date.

IL-1β was detectable in 96 out of 113 samples (range 0 – 9580 pg/ml). The median concentration of IL-1β in samples obtained from women with bacterial vaginosis (640.3 pg/ml; interquartile range 258.3-1120) was significantly higher than that of samples obtained from women with normal flora (195.3 pg/ml; interquartile range 93.6-465.4) ($P<0.01$; Figure 3.5).

IL-1β's antagonist IL1RA was detectable in all 113 samples of cervicovaginal secretions, at much higher concentrations than IL-1β (range 15.6 – 7566 ng/ml). The median concentrations of IL1RA in samples obtained from women with bacterial vaginosis (730.4 ng/ml; interquartile range 447.5-1304) and intermediate flora (658.9 ng/ml; interquartile range 398.5-851.9) were both significantly higher than those of samples obtained from women with normal flora (257.8 ng/ml; interquartile range 27.3-425.8) ($P<0.01$ and $P<0.05$; Figure 3.6).

IL-8 was detectable in 109 out of 113 samples of cervicovaginal secretions (range 0-17362 pg/ml). There were no significant differences in the median concentrations of IL-8 in samples obtained from women with normal flora (1748 pg/ml; interquartile

range 839.2-3924) intermediate flora (2176 pg/ml; interquartile range 754-4346) or bacterial vaginosis (3318 pg/ml; interquartile range 261.4-6655) (P=0.49; Kruskal-Wallis test).

CCL5/RANTES were not detectable in any of 18 cervicovaginal secretion samples tested (6 each from women with normal flora, intermediate flora and bacterial vaginosis).

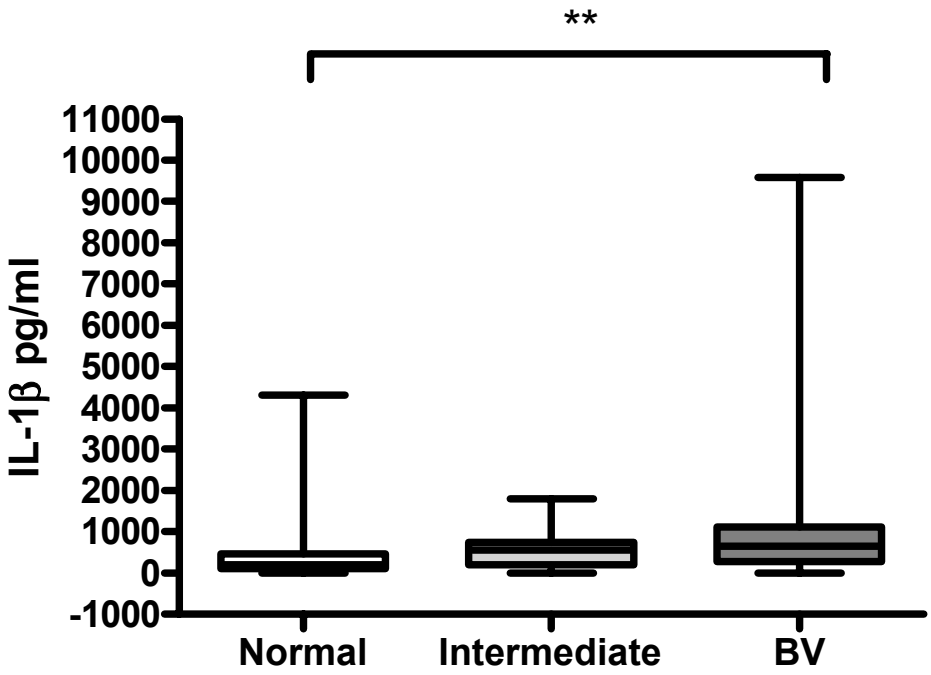


Figure 3.5

Median concentrations of IL-1β (pg/ml) +/- interquartile range (box) and range (whiskers) in cervicovaginal secretions obtained from women in the second trimester of pregnancy with normal vaginal flora (n=66), intermediate vaginal flora (n=20), or bacterial vaginosis (n=27). **P<0.01 determined by Kruskal-Wallis test.

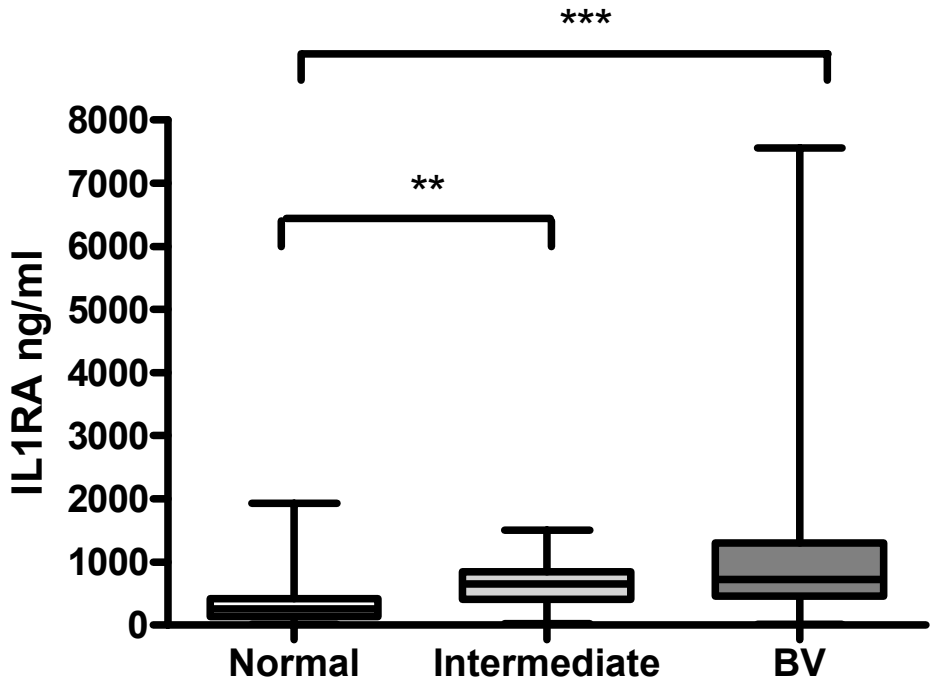


Figure 3.6

Median concentrations of IL-1RA (ng/ml) +/- interquartile range (box) and range (whiskers) in cervicovaginal secretions obtained from women in the second trimester of pregnancy with normal vaginal flora (n=66), intermediate vaginal flora (n=20), or bacterial vaginosis (n=27). **P<0.01 and P<0.001 determined by Kruskal-Wallis test.

3.3.6. Natural antimicrobial production by vaginal, ectocervical and endocervical cell lines

Expression of natural antimicrobial mRNA was compared in vaginal keratinocyte VK2 E6/E7 (VK2), ectocervical ECT E6/E7 (ECT) and endocervical END E6/E7 (END) cell lines using Taqman PCR.

VK2 cells expressed significantly more elafin mRNA than ECT or END cells ($P<0.001$), whereas no differences in expression of SLPI, HBD1 or HBD3 mRNA were detected between the cell lines (Figure 3.7). HBD2 was detected at very low levels in VK2 cells, but was found in only one sample out of three from each other cell line. HBD4 was not expressed in any of the cells (data not shown).

The secretion of elafin, SLPI, HBD2 and HBD3 was assessed in each cell type by performing ELISA on cell media. At the time of experimentation, no reliable assay for HBD1 was available. Cells were grown to confluence, and then washed twice to remove any adherent natural antimicrobials. Fresh media was added and immediately removed for assay (timepoint 0 hours). This was done in order to ensure results represented natural antimicrobial secretion by an intact monolayer of cells as opposed to those secreted whilst the cells were growing to confluence, or any inherent in the media. Fresh media was added and cells cultured for 24 hours, at which point media was again harvested for assay (timepont 24 hours).

There was detectable secretion of elafin by VK2 and END cells at 24 hours ($P<0.001$), but there was no detectable production by ECT cells (Figure 3.8 A). SLPI was secreted by all three cell lines ($P<0.001$; Figure 3.8 B). HBD2 was secreted at much lower levels, with significant production at 24 hours by VK2 cells only ($P<0.01$; 3.8 C). HBD3 was undetectable in all three cell lines.

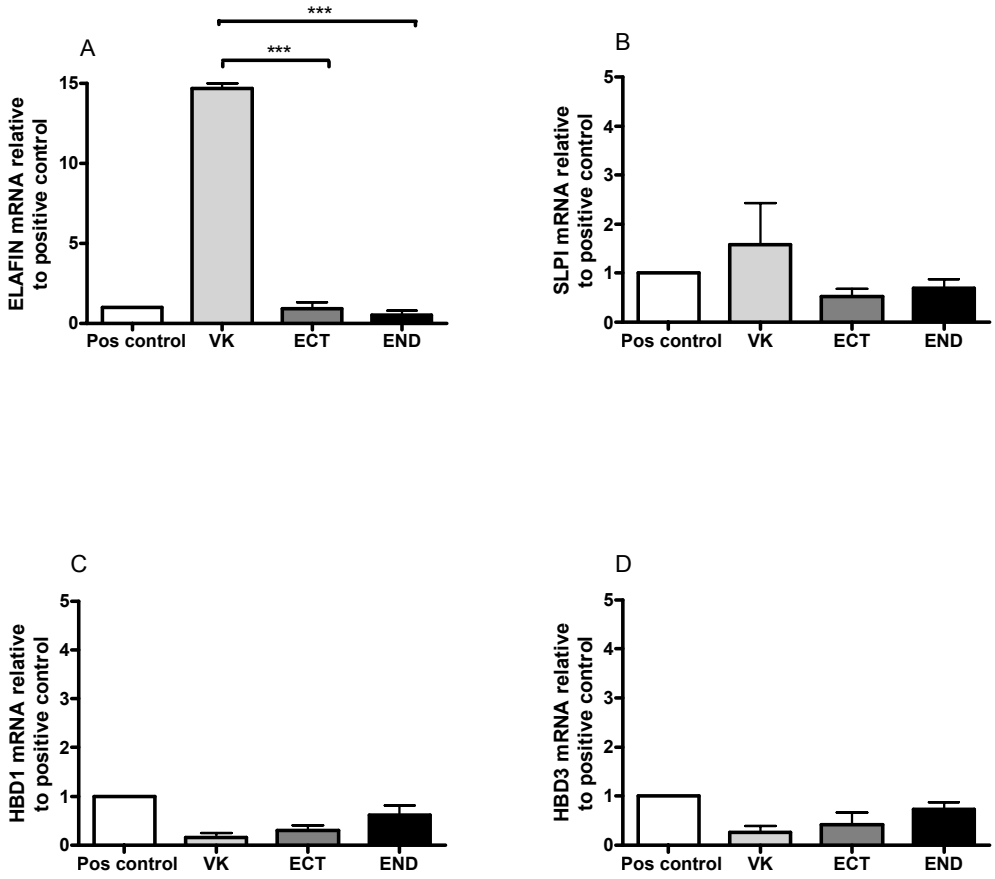


Figure 3.7

Mean +/- SEM expression of natural antimicrobial mRNA in VK (n=3), ECT (n=3) and END (n=3) cells, relative to positive control by Taqman qPCR. A=Elafin, B=SLPI, C=HBD1, D=HBD3. ***P<0.001 (One-way ANOVA with Tukey's post-test).

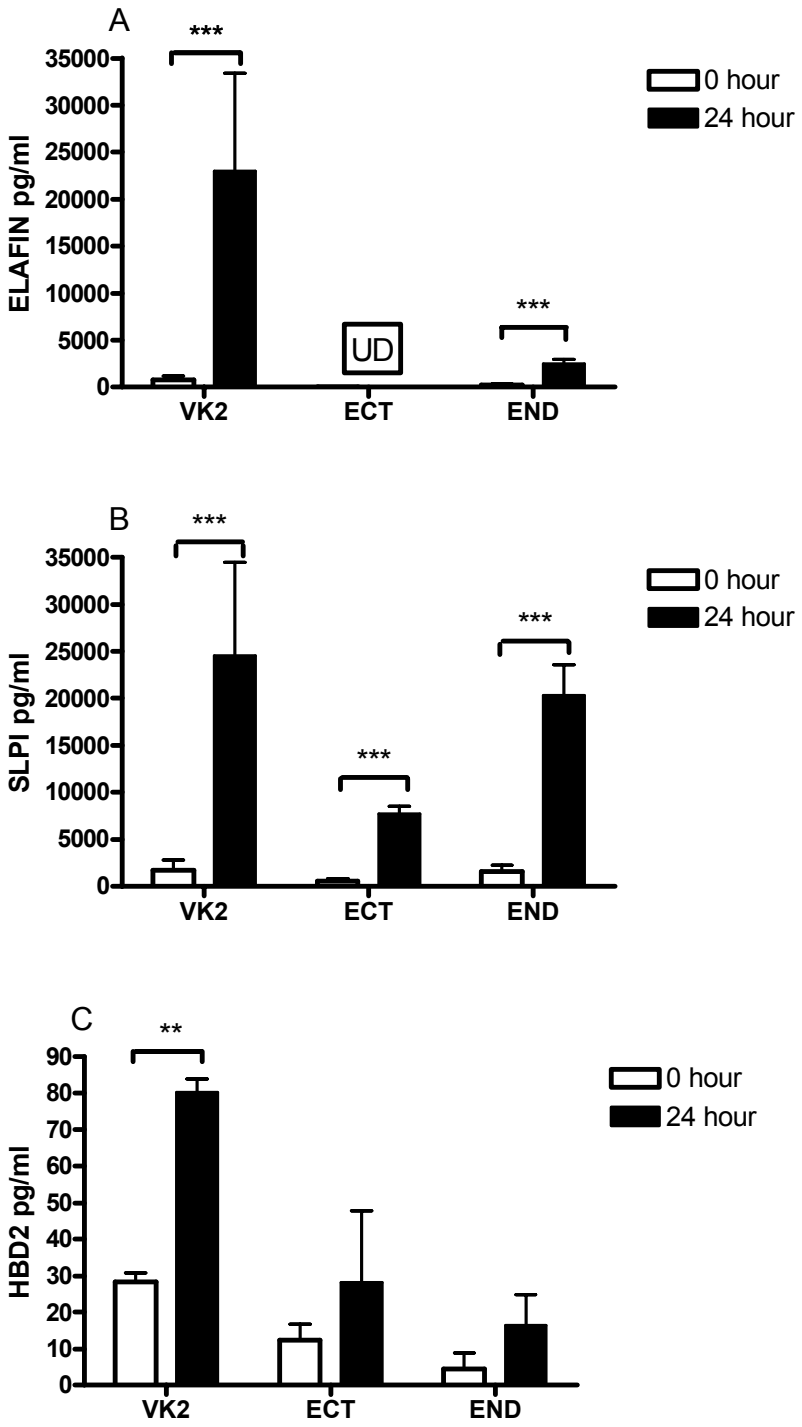


Figure 3.8

Mean +/- SEM amount of elafin (A), SLPI (B) and HBD2 (C) (pg/ml) secreted by VK2 (n=3), ECT (n=3), and END (n=3) cells obtained at 0 and 24 hours, as measured by ELISA. UD= undetectable. **P<0.01 ***P<0.001 (Paired t-test).

The effect of LPS, LTA or IL-1ß stimulation (duration 24 hours) on production of elafin, SLPI and HBD2 was investigated in each cell type.

3.3.6.1. *Elafin*

Production of elafin mRNA (Taqman qPCR) and protein (ELISA) by VK2 cells was unaffected by any of the treatments (Figure 3.9 A and B). LTA decreased expression of elafin mRNA; and LPS and IL-1ß increased expression of elafin mRNA in ECT cells approximately 2-fold ($P<0.05$; Figure 3.9 C). However ECT cells did not secrete detectable amounts of elafin protein, even after stimulation (Figure 3.9 D). In END cells LPS significantly upregulated expression of elafin at both mRNA and protein level ($P<0.05$ and $P<0.001$; Figure 3.9 E and F).

3.3.6.2. *SLPI*

No treatment affected expression of SLPI mRNA in any cell lines (Figure 3.10 A, C and E). Nonetheless, LPS and IL-1ß increased secretion of SLPI protein by END cells ($P<0.05$; Figure 3.10 F)

3.3.6.3. *HBD2*

Expression of HBD2 mRNA in unstimulated cells was too low to give meaningful results using Taqman PCR. There was a trend for increased production of HBD2 by VK2 cells upon stimulation with IL-1ß, but this did not reach statistical significance (data not shown).

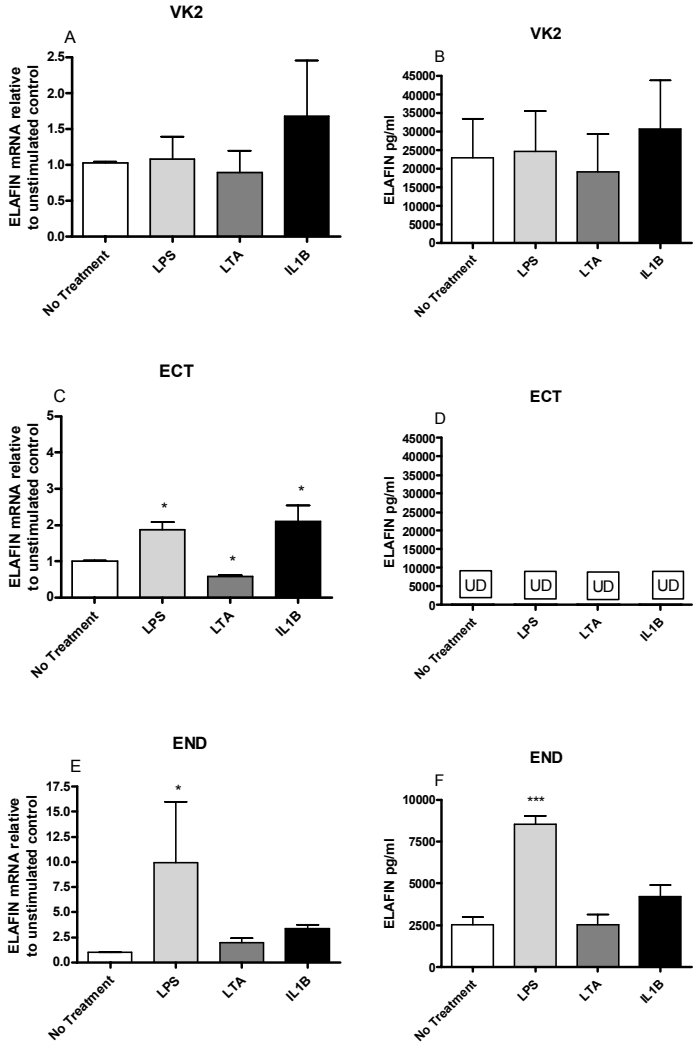


Figure 3.9

Mean +/- SEM expression of elafin mRNA relative to untreated control determined by Taqman PCR (Figures A, C and E) and elafin protein in pg/ml determined by ELISA (Figures B, D and F) in VK (n=3), ECT (n=3) and END (n=3) cells, following 24 hours stimulation with LPS (1µg/ml), LTA (500ng/ml) or IL-1ß (10ng/ml). UD= undetectable. *P<0.05 and ***P<0.001 (One-way repeated measures ANOVA with Tukey's post-test).

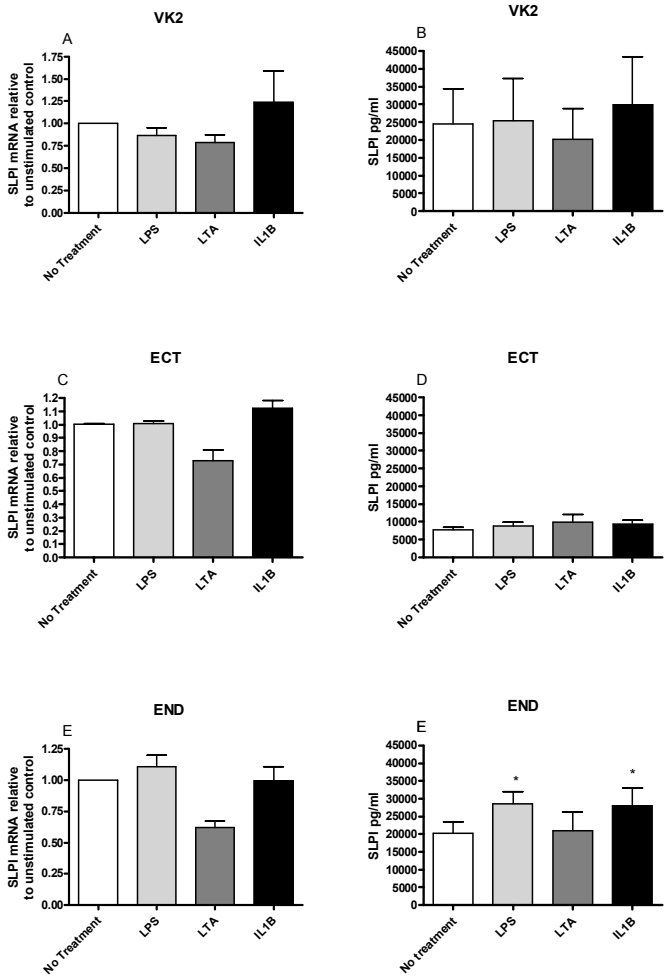


Figure 3.10

Mean +/- SEM expression of SLPI mRNA relative to untreated control determined by Taqman PCR (Figures A, C and E) and SLPI protein in pg/ml determined by ELISA (Figures B, D and F) in VK (n=3), ECT (n=3) and END (n=3) cells, following 24 hours stimulation with LPS (1µg/ml), LTA (500ng/ml) or IL-1ß (10ng/ml). *P<0.05 (One-way repeated measures ANOVA with Tukey's post-test).

3.3.7. Cytokine production by vaginal, ectocervical and endocervical cell lines

Expression of IL-1ß, IL1RA and IL-8 mRNA was compared in VK2, ECT and END cell lines using Taqman PCR.

VK2 cells expressed more IL-1ß mRNA than ECT ($P<0.05$) or END ($P<0.01$) cells, but levels of IL1RA mRNA were similar in all three cell lines (Figure 3.11 A and B). Expression of IL-8 mRNA was lower in VK2 cells than in ECT cells ($P<0.05$; Figure 3.11 C).

Stimulation of the three cell lines with LPS, LTA or IL-1ß for 24 hours did not significantly effect expression of IL-1ß, IL1RA or IL-8 mRNA (data not shown).

3.3.8. The effect of progesterone on natural antimicrobial and cytokine production by vaginal and cervical cell lines

Treatment of VK2, ECT and END cells with progesterone 1µM for 24 hours did not significantly affect mRNA expression of SLPI, elafin, HBD1, HBD3, IL-1ß, IL1RA or IL-8, as determined by Taqman qPCR (data not shown).

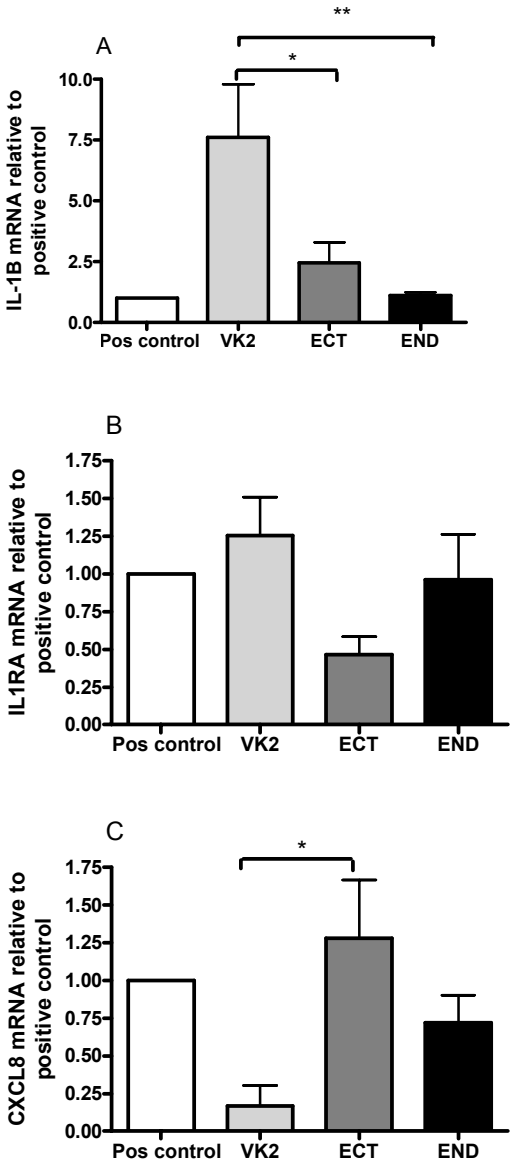


Figure 3.11

Mean +/- SEM expression of cytokine mRNA in VK (n=3), ECT (n=3) and END (n=3) cells, relative to positive control determined by Taqman qPCR. A= IL-1ß, B=IL1RA, C=IL-8. *P<0.05 and **P<0.01 (One-way ANOVA with Tukey's post-test).

3.4. DISCUSSION

Natural antimicrobials in cervicovaginal secretions

This chapter showed that natural antimicrobial proteins are a component of cervicovaginal secretions. SLPI and elafin were consistently present, and found at much higher concentrations than HBD2 or HBD3. Both antileukoproteinases were found in sufficient amounts to contribute to the microbiocidal capacity of cervicovaginal fluid. SLPI has been previously reported in cervical secretions (Kramps, Franken et al. 1984). It is also found at extremely high levels in the cervical mucus plug in pregnancy, and levels of SLPI are 10-12 fold higher in cervicovaginal secretions in pregnancy than in the non-pregnant state (Helmig, Uldbjerg et al. 1995). Elafin has been immunolocalized in the vaginal epithelium (Pfundt, Wingens et al. 2000) but this is the first report of its presence in cervicovaginal secretions.

In this study levels of both SLPI and elafin were found to be lower in women with bacterial vaginosis, but there was no evidence of changes in defensin levels. The finding that levels of SLPI are decreased in association with bacterial vaginosis corroborates those of Draper et al. They found that cervicovaginal concentrations of SLPI are decreased in the presence of genital tract infections, including bacterial vaginosis, in both pregnant and non-pregnant women (Draper, Landers et al. 2000). Another study in non-pregnant women found decreased SLPI in cases of bacterial vaginosis diagnosed by Amsel's criteria (Valore, Wiley et al. 2006). However, this study also found diminished levels of HBDs associated with bacterial vaginosis, which differs from the findings in this chapter. Samples were collected by vaginal washout with water and defensins measured by immunoblot assay, and these conflicting results may be a result of different research methodology. Alternatively, they could reflect variations in cervicovaginal innate immunity in the pregnant and non-pregnant states, which is an area for upcoming investigation.

The deficiency in SLPI and elafin in bacterial vaginosis may result from the change in bacterial flora. In the mouth, commensal bacteria induce the expression of HBD2 (Krisanaprakornkit, Kimball et al. 2000), and a similar mechanism may exist in the

lower genital tract. Thus the decrease in numbers of lactobacilli, the normal commensal of the vagina, seen in bacterial vaginosis may reduce the stimulus for natural antimicrobial production. The resultant innate immunosuppression could predispose to the establishment of other infections, including HIV and those that can cause preterm labour. Lactobacilli have been shown to stimulate HBD2 protein in vaginal cells cultured with dendritic cells (Valore, Wiley et al. 2006). The phenomenon has not been described with either SLPI or elafin, but the diverse nature of vaginal flora in both healthy women and those with bacterial vaginosis complicates its study.

Alternatively, the deficiency in SLPI and/or elafin may precede bacterial vaginosis, rather than being a consequence of the infection. Both SLPI and elafin have activities against Gram-positive and Gram-negative bacteria (Hiemstra, Maassen et al. 1996; Simpson, Maxwell et al. 1999). If they form an integral part of cervicovaginal defences, a deficit may predispose to the overgrowth of pathogenic bacteria. Genetic polymorphisms could underlie such a deficiency, by causing a generalized decrease in innate defences. In this case, bacterial vaginosis could represent a global vulnerability to infections, including those that cause preterm labour, rather than directly linking the conditions. This could also explain the observation that periodontal infection is associated with higher rates of preterm delivery. Valore et al (Valore, Wiley et al. 2006), found a return to normal cervicovaginal levels of SLPI after treatment of bacterial vaginosis. This makes a deficiency in constitutive production less likely, although does not exclude the possibility of an attenuated response to infectious agents or cytokines.

HBD3 has not been described in the lower genital tract before. It was absent in the majority of samples of cervicovaginal secretions examined, or found only at low levels. HBD2 was also found at relatively low concentrations. This is in agreement with findings in vaginal secretions in non-pregnant women (Valore, Park et al. 2002). In optimal laboratory conditions beta-defensins are active at low micro molar concentrations (Ganz 2003), but activity deteriorates with increasing salt concentrations and plasma proteins. It seems unlikely that the beta-defensins would

have much antimicrobial capability in cervicovaginal secretions at the concentrations found. However, they are highly basic proteins, which tend to be intimately associated with epithelial surfaces. They thus may be present locally at high enough concentrations to effect microbial neutralization, but this remains difficult to prove.

Bacterial vaginosis is associated with a high pH, secondary to the deficiency in lactobacilli which may influence natural antimicrobial attachment to tissues, but may also have other consequences. A recent study has shown that HBDs have more effective antibacterial activity in acidic environments (Chen, Niyonsaba et al. 2005). This implies that bacterial vaginosis may be associated with decreases in natural antimicrobial function, further promoting bacterial overgrowth and/or secondary infections.

Cytokines in cervicovaginal secretions

Cytokine levels in cervicovaginal secretions were also examined with relation to bacterial vaginosis. Bacterial vaginosis is generally thought of as a non-inflammatory condition. Nevertheless, levels of the inflammatory cytokine IL-1ß are increased in cervical and vaginal secretions when the condition is present (Imseis, Greig et al. 1997; Mattsby-Baltzer, Platz-Christensen et al. 1998; Spandorfer, Neuer et al. 2001; Cauci, Guaschino et al. 2003; Basso, Gimenez et al. 2005; Hedges, Barrientes et al. 2006; Valore, Wiley et al. 2006). The results of this study also show this. There was however, no evidence of an associated rise in IL-8, which is the major chemotactic factor for neutrophils. It has been speculated that in bacterial vaginosis there is microbial inhibition of IL-8 production in response to IL-1ß, although the mechanism for this is not clear (Cauci, Guaschino et al. 2003). The diminished response may explain why the number of vaginal polymorphonuclear cells found in bacterial vaginosis is not increased with respect to that in healthy women (Cauci 2004; Ramsey, Lyon et al. 2005). CCL5/RANTES is another neutrophil chemotactin, which is reported as being produced by cervical cells (Fichorova and Anderson 1999). This was undetectable in all samples of cervicovaginal secretions examined.

There is conflicting data regarding the response of IL-1ß's antagonist IL1RA in bacterial vaginosis. Genc et al found increased IL1RA levels in bacterial vaginosis (Genc, Vardhana et al. 2004), whereas Valore et al didn't detect a significant difference (Valore, Wiley et al. 2006). In this study IL1RA was raised in women with both bacterial vaginosis, and in women with intermediate flora. It has been suggested that a disproportionate rise in IL-1ß in comparison to IL1RA is associated with vaginal leucocytosis (Donders, Bosmans et al. 2000), and preterm delivery (Genc, Vardhana et al. 2004). In this study the IL1RA: IL-1ß ratio was not different in any group (data not shown).

Sample collection and diagnosis of bacterial vaginosis

Determining the prevalence of bacterial vaginosis was not an aim of this study. The sample group was too small for accurate evaluation of the prevalence of such a common condition, which should be carried out in a much larger cohort. Similarly, correlation of pregnancy outcome with bacterial vaginosis status or natural antimicrobial levels is not valid in such a small sample group, and this data was provided for completeness.

With this proviso, the incidence of bacterial vaginosis found was surprisingly high at 24.1%. This is lower than 33% found in an inner city group in the USA (McGregor, French et al. 1995) but higher than the 15% found in a middle class area in London (Hay, Morgan et al. 1994). Consideration has to be given to possible reasons for this. Incidence of bacterial vaginosis is higher in early pregnancy than in later pregnancy or general gynaecology populations, when most large studies in the UK have been performed. Nevertheless, a recent Danish study in 3540 predominantly Caucasian women under 20 weeks gestation found a prevalence of bacterial vaginosis of only 17% (Svare, Schmidt et al. 2006). Here bacterial vaginosis was diagnosed using the less well-established Schmidt method that uses unstained vaginal smears. Only one study has been performed comparing the Schmidt and the Nugent diagnostic methods (Schmidt and Hansen 2001). This suggested that Nugent's criteria might give a higher rate of false positives than the Schmidt method of diagnosis, which could explain the differences in prevalence seen. However, Nugent's method was chosen in

our study as it has been validated and is extensively used for clinical diagnosis and in research (Nugent, Krohn et al. 1991; Hillier, Krohn et al. 1992; Tam, Yungbluth et al. 1998). It is recognized that a large-scale comparison of diagnostic methods is desirable, and standardized methods should be used in subsequent research to ensure the greatest applicability of the work.

Samples of cervicovaginal secretions were collected from pregnant women below 20 weeks gestation. Sample collection was carried out at this time as there is some evidence that bacterial vaginosis occuring early in pregnancy is more likely to be associated with poor pregnancy outcome (Leitich, Bodner-Adler et al. 2003). Cervicovaginal secretions were self-collected by tampon insertion which avoided the need for speculum examination. This is less time and resource consuming in busy community antenatal clinics and may be more acceptable to women and their attendant midwives. The method has been validated for the diagnosis of bacterial vaginosis (Sturm, Moodley et al. 2002) and a similar method has been successfully used in a previous study of components of vaginal fluid (Valore, Park et al. 2002). Unfortunately it was not possible to diagnose other genital tract infections in this study. Although it is likely that the rates of such infections are low in the population studied, these could be a confounding factor on results.

A problem with the method of sample collection used is that the amount of cervicovaginal secretions obtained may vary and the decreased amount of SLPI and elafin seen in women with bacterial vaginosis may merely represent smaller sample volumes obtained from these women. In order to minimize variation, identical tampons were used throughout the study, and inserted for a standard time (15 minutes). Initially tampons were weighed pre and post specimen collection to estimate sample volume so levels could be expressed per mililitre, but this was found to be neither accurate nor practical. Instead the amount of total protein in each sample was ascertained, and it was anticipated that this could be used to standardize results by expressing the amount of the substance of interest as a proportion of the amount of total protein. However, it was found that the amount of total protein obtained from women with bacterial vaginosis was lower than that obtained from

women with normal flora. This agrees with results from a study in non-pregnant women where the authors suggest that in bacterial vaginosis the overgrown bacteria metabolize cervicovaginal proteins (Valore, Wiley et al. 2006) and if true invalidates total protein as a measure of sample volume. Nonetheless when total protein was used as a denominator for natural antimicrobial and cytokine levels, similar patterns were seen (data not shown). The trend for lower natural antimicrobial levels in women with bacterial vaginosis was less pronounced, and although SLPI was still significantly decreased the decrease in elafin was not significant. The increase in cytokines was more apparent.

A possible influence on sample volume is pH. More basic protein may be available at the low pHs found in vaginas with healthy flora, than at the higher pHs found in bacterial vaginosis. In our sampling method tampons physically dislodged samples from the walls of the vagina, so it is less likely that pH would have a major influence. In addition, the similar cytokine patterns seen in this study and those of others add credence to the method of specimen collection. Nonetheless, the effect of pH is worth further investigation, and sampling with acidified medium should be considered in future research.

In this study, the vast majority of participants were Caucasian, reflecting the population in Edinburgh. The groups of women with bacterial vaginosis, intermediate flora and normal flora were comparable in age and gestation, however there were more smokers in the group with bacterial vaginosis. This is not surprising as smoking is an independent risk factor for bacterial vaginosis, exhibiting a dose response effect, although the reasons for this are not clear (Hellberg, Nilsson et al. 2000). The amines cotinine and nicotine are concentrated in the cervical mucus of smokers, (McCann, Irwin et al. 1992), but the biological relationship of these to the development of bacterial vaginosis has not been studied. It is possible that they may alter the pH of the lower genital tract, predisposing to change in bacterial flora. The association between smoking and natural antimicrobial levels has also not been investigated. The numbers in this study are too small to give valid analysis in this respect. It is interesting to note however, that levels of HBD2 seemed to be higher in

smokers than non-smokers (data not shown), and this would make an interesting area for further study.

In vitro natural antimicrobial production

Cervicovaginal fluid is a mixture of secretions from the vagina, and the ectocervical and endocervical glands. *In vitro* experiments were performed in order to further investigate the production of natural antimicrobials and cytokines by the individual epithelia. Although primary cell cultures would be desirable for this, tissues for the development of cultures from the vagina and cervix are not readily obtainable. Instead human papillomavirus E6/E6E7 immortalized vaginal, ectocervical and endocervical epithelial cell lines were used. These have been shown to maintain a phenotype similar to primary cell cultures of the tissues of origin (Fichorova, Rheinwald et al. 1997). In addition these cell lines have been derived from the same patient, using the same immortalization protocol (Fichorova, Rheinwald et al. 1997) and they were cultured under identical conditions. This minimizes variation secondary to genetics and methodology, allowing a more valid comparison of the antimicrobial and cytokine production of the different cell types.

Vaginal and endocervical cell lines exhibited different profiles of elafin, SLPI and cytokine production. The vagina is host to a diverse microflora, and tolerant to a variety of bacteria and foreign antigens. In contrast the endocervix is the normally sterile channel to the upper genital tract, where microbial penetration can be extremely detrimental. It thus seems logical that the two areas would have different innate immune responses – a theory supported by results of the cell line experiments. Under basal conditions the vaginal cell line expressed more elafin than the ecto or endocervical cell lines. In animal models elafin has been shown to inhibit inflammatory infiltration in arterial and lung wall (Zaidi, You et al. 2000; Vachon, Bourbonnais et al. 2002), and can also inhibit NFκB and AP-1 activation (Henriksen, Hitt et al. 2004; Butler, Robertson et al. 2006). Thus elafin may have an anti-inflammatory role in the lower genital tract. The vaginal cell line also exhibited lower expression of the neutrophil attractant IL-8 mRNA, although there was higher expression of IL-1ß. Lack of vaginal neutrophil infiltration in bacterial vaginosis is

well recognised, despite the presence of pathogenic organisms (Cauci 2004; Ramsey, Lyon et al. 2005) and low local expression of IL-8 could contribute to this.

There was no change in expression of any natural antimicrobial or cytokine in the vaginal cell line upon LPS, LTA or IL-1ß stimulation, suggesting some innate immune tolerance in this tissue. The mechanism of this is unclear. There is conflicting evidence regarding vaginal expression of TLR4 (Fazeli, Bruce et al. 2005; Pivarcsi, Nagy et al. 2005), however TLR4 mRNA was detected in VK2 cells. In addition CD14 mRNA was expressed. In the intestine, which also hosts a diverse microflora, a variety of mechanisms have been recognized which promote microbial tolerance (Cario and Podolsky 2005). These include alterations in cellular localization of TLRs and the interactions of negative adaptor proteins or co-factors such as TOLLIP. Investigation of similar mechanisms in the genital tract is warranted.

LPS did affect the endocervical cell line. Both elafin mRNA and protein was upregulated in response to LPS in endocervical cells. In addition SLPI protein was increased, although there was no increase in SLPI mRNA expression evident, suggesting that this may be a result of release of stored SLPI rather than *de novo* synthesis.

The upregulation of elafin and SLPI at the endocervix in response to inflammatory stimuli could serve several purposes. They are directly antimicrobial, and may help to eliminate pathogens from the normally sterile area (Simpson, Maxwell et al. 1999; Sallenave 2002). SLPI and elafin also exhibit some pro-inflammatory activities, and LPS induced inflammatory cellular infiltration is increased in murine lung expressing the human elafin gene (Simpson, Cunningham et al. 2001; Sallenave, Cunningham et al. 2003). This suggests they may enhance the protective innate immune response at the site of microbial invasion. In comparison to the vaginal epithelium, the endocervix is lined by a single layer of columnar epithelium thus is more susceptible to disruption. The potent protease inhibitor activity of SLPI and elafin could decrease tissue damage from human neutrophil elastase and other proteases released

at the sight of inflammation (Sallenave, Si Tahar et al. 1997; Simpson, Wallace et al. 2001). In monocytes SLPI inhibits LPS induced MMP production and PGE2 via inhibition of PGHS-2 (Zhang, DeWitt et al. 1997); both molecules are crucial in the remodelling of the cervix in labour, and uncontrolled release could lead to preterm delivery. Finally, the endocervix is the primary site of plasma and T cell localization in the lower genital tract (Anderson, Davidson et al. 1996). In overwhelming infection SLPI and elafin produced here may be involved in the recruitment and modulation of the adaptive immune response (Roghanian, Williams et al. 2006). The production of SLPI and elafin in the genital tract requires further investigation, which must include consideration of the interactions between the epithelial cells, other inflammatory cells and stromal cells. They possess many functions that could be relevant in reproductive biological processes, and in the long term may even have therapeutic potential as modulators of infection and inflammation.

The ectocervical and endocervical cell lines had similar patterns of unstimulated natural antimicrobial and cytokine secretion. However, there was little evidence of a response to LPS in the ectocervical cells, making it more similar to the vaginal cell line than the endocervical cell line in this respect. This phenotype reflects its position, as the external portion of the cervix, which is also under constant exposure to bacteria and antigens.

HBD expression was low in all the cell lines, with HBD3 protein production undetectable in all three cell lines, and only low level secretion of HBD2 by the vaginal cell line. This corresponds with our findings in cervicovaginal secretions. The low mRNA expression of HBD2 made it impossible to accurately assess the effect of stimuli on the cells using Taqman PCR. Nevertheless, examination of protein secretion did not indicate any significant effect of LPS, LTA or IL-1ß on HBD2 production. This finding is supported by those of a previous report which showed vaginal expression of the mouse beta-defensins 1, 2 and 4 are not affected by bacterial products (Soboll, Schaefer et al. 2006). However, another study, which used a different immortalized vaginal cell line, showed HBD2 was secreted and expression was significantly upregulated by the microbial products LPS, PG and

heat-killed *Candida albicans* (Pivarcsi, Nagy et al. 2005). These divergent findings may reflect differences in the cell types used.

There is some evidence that progesterone can influence the production of SLPI and other natural antimicrobials. They are expressed cyclically in the endometrium (King, Critchley et al. 2000; King, Fleming et al. 2003) and can be modulated by hormonal contraception (Fleming, King et al. 2003). Progesterone also upregulates expression of SLPI mRNA in a breast epithelial cell line, and increases secretion of SLPI by cervical explants (Denison, Calder et al. 1999). Expression of natural antimicrobial mRNA by vaginal, ectocervical, and endocervical cell lines was, however, unchanged by 24 hours of progesterone treatment. Cytokine expression was similarly unaffected, despite progesterone receptor mRNA expression in all the cell lines being confirmed by Taqman PCR. This may indicate that progesterone is not a major influence on the innate immune responses of the lower genital tract.

Additional *in vivo* assessment of the effect of progesterone was planned, through the analysis of cervicovaginal secretion samples collected from women who were enrolled on the "STOPPIT" trial (https://www.charttrials.abdn.ac.uk/stoppit/). This is a multicentre double blind randomised placebo controlled study of daily vaginal progesterone gel (90mg) from 24 -34 weeks gestation, for the prevention of preterm birth in twins. Samples were collected from participants in Edinburgh once whilst on the trial, and once after the intervention had been stopped. Due to overall slow recruitment to the trial, it has now been extended, and unblinding will not occur until 2008. This means analysis of the results could not be included as part of this thesis.

Summary

In summary, this chapter provides further evidence that the lower genital tract has an active innate immune system, capable of producing natural antimicrobials, chemokines and cytokines. The study of cell lines has suggested divergent responses of vaginal and cervical epithelia, reflecting their differing functions. Elafin has, for the first time, been identified as a component of cervicovaginal secretions. Levels of both elafin and SLPI were diminished in bacterial vaginosis, signifying potential

involvement in either the pathogenesis of the infection, or its sequelae which include preterm labour and increased HIV transmission. Their role was speculated on, and further study of these functions could broaden understanding of inflammatory processes in the genital tract that can have devastating consequences on fertility and pregnancy.

4. Natural antimicrobial expression in the amnion

4.1 INTRODUCTION

The fetal membranes require competent defences to protect against pathogens that can jeopardise the pregnancy. Natural antimicrobials are essential components of the innate immune system (Ganz 2003) with broad-spectrum activities against bacteria, yeasts and some viruses (Bals 2000). They provide a complementary chemical barrier to the physical barrier properties of epithelial surfaces. Their production by the innermost fetal membrane, the amnion, was investigated in this chapter.

The bacterial product lipopolysaccharide (LPS) has been shown to stimulate HBD3 mRNA in so-called amnion derived FL cells (Buhimschi, Jabr et al. 2004). However, this cell line has now been shown to be derived from cervical He-La cell contaminants (Nelson-Rees and Flandermeyer 1976) (www.atcc.org), thus the validity of this finding is unclear. The other commercially available "amnion" cell line, WISH, is similarly contaminated (Kniss, Xie et al. 2002) (www.atcc.org). In this chapter natural antimicrobial expression in primary cultured amnion cells and He-La cells was compared to expression in the FL and WISH cell lines, to clarify whether these are a suitable model for studying the innate immune response in the amnion.

Natural antimicrobial production was also examined in amnion tissue in labour and non-labour settings. Labour is an inflammatory process, mediated by leukocyte invasion of the fetal membranes, and increased cytokine concentrations (Kelly 1996; Bowen, Chamley et al. 2002; Keelan, Blumenstein et al. 2003; Yellon, Mackler et al. 2003). In many tissues natural antimicrobial expression is stimulated by bacterial products and inflammatory cytokines such as LPS, IL-1β and TNF-α (Singh, Jia et al. 1998; Mathews, Jia et al. 1999; O'Neil, Porter et al. 1999; Garcia, Krause et al. 2001; Harder, Bartels et al. 2001; King, Fleming et al. 2002; Tsutsumi-Ishii and Nagaoka 2003), and the role of these mediators on natural antimicrobial production in the amnion was explored. In addition, the effects of IL-17, and dexamethasone were assessed. IL-17 is a T-cell cytokine which is a potent inducer of the natural antimicrobial HBD2 in tracheobronchial epithelium (Kao, Chen et al. 2004).

Dexamethasone has been shown to influence HBD2 expression in intestinal epithelial cell lines (Witthoft, Pilz et al. 2005).

The aim of this research was to determine natural antimicrobial expression in the amnion and establish the effect of inflammatory cytokines on their production. Natural antimicrobials may be an important component of the inflammatory response associated with parturition. It was anticipated that results of these investigations could contribute to our understanding of this complex process.

4.2 METHODS

All materials, reagents and cell lines are detailed in Appendix 1.

4.2.1 Specimen Collection

Samples were collected as described in Section 2.1.2. The samples used in the experimental work in this chapter are shown below (Table 4.1).

Tissue	Experimental Use	No
Prelabour amnion (Elective LSCS)	Primary amnion epithelial cell culture	30
Prelabour amnion (Elective LSCS)	Amnion explant culture	4
Prelabour amnion (Elective LSCS)	RNA extraction and Taqman PCR	8
Prelabour choriodecidua (Elective LSCS)	RNA extraction and Taqman PCR	3
Prelabour placenta (Elective LSCS)	RNA extraction and Taqman PCR	3
Postlabour amnion (SVD)	RNA extraction and Taqman PCR	7
Endometrium(menstrual phase)	RNA extraction and Taqman PCR	1
Endometrium(proliferative phase)	RNA extraction and Taqman PCR	1
Endometrium(midsecretory phase)	RNA extraction and Taqman PCR	1

Table 4.1
Patient samples used in experiments in Chapter 4

4.2.2 *In Vitro* culture

4.2.2.1 *Primary amnion epithelial cell culture*

Primary amnion epithelial cells were cultured as detailed in Section 2.2.1.1.

Cells were treated with recombinant human IL-1β 0.01-100ng/ml; TNFα 10ng/ml; IL-17 10ng/ml; LPS 1μg/ml; dexamethasone 1μM and/or IL-1β 10ng/ml; or equivalent volumes of vehicle (serum free media for cytokines; ethanol for dexamethasone). Doses were indicated by previous published reports TNFα, IL-1β and LPS (Krisanaprakornkit, Kimball et al. 2000); IL-17 (Kao, Chen et al. 2004) and dexamethasone (Witthoft, Pilz et al. 2005)]. In addition IL-1β dose response experiments were carried out (see results). Treatments were applied for 0, 1, 2, 3, 6, 12, 16, 24, or 48 hours as detailed in results.

Experiments were performed in quadruplicate. RNA was extracted from one set of duplicates for analysis of natural antimicrobial expression by Taqman quantitative PCR (Section 2.3-4). Media was harvested from the other set of duplicates, stored at -20°C and analyzed by HBD2 ELISA (Section 2.5.1). The remaining cells were lysed for total cellular protein quantification (Section 2.5.9).

4.2.2.2 *FL/WISH/He-La cell culture*

FL/WISH and He-La cells were cultured as detailed in Section 2.2.1.2.

4.2.2.3 *Amnion Explant culture*

12mm amnion explants were cultured as detailed in Section 2.2.2. Explants were treated with recombinant human IL-1β 10ng/ml or an equivalent volume of serum free media, for 24 hours. Media was harvested, stored at -20°C and analyzed by HBD2 ELISA (Section 2.5.1). Explants underwent RNA extraction for analysis of natural antimicrobial RNA expression by Taqman quantitative PCR (Section 2.3-4).

4.2.3 Tissue RNA extraction

RNA was extracted from fresh amnion, choriodecidua and placental samples as detailed in Section 2.3.1.2, and analysed for natural antimicrobial expression by Taqman quantitative PCR (Section 2.3).

4.2.4 Statistical Analysis

Taqman quantitative PCR data was analyzed using the ΔΔCT method as described in Section 2.4.2. In experiments designed to assess the effects of treatments, where treated samples were related to untreated controls, normalizing the untreated data to a value of 1 invalidates subsequent statistical analysis. Statistical analysis was therefore performed on the ΔCT values of samples (where ΔCT is the difference between the cycle threshold values of the amplicon of interest and 18s). In experiments where all samples were related to the same positive control, this was not necessary, and analysis was performed on the final relative values.

The distribution of data was determined using the Kolmogorov-Smirnov test for normality. Parametric data was analyzed using the paired t-test (2 groups) or one-way ANOVA and Tukey's test to assign individual differences (3 or more groups). Repeated measure ANOVA was used when appropriate. Graphs of parametric data represent mean +/- standard error of the mean (SEM). Non-parametric data was analyzed using the Friedman test. Graphs of non-parametric data represent median +/- interquartile range. $P<0.05$ was regarded as significant.

4.3 RESULTS

4.3.1 Natural antimicrobial expression in primary cultured amnion epithelial cells is different from that in FL, WISH and He-La cell lines.

Primary cultured amnion cells expressed HBD1, HBD2, HBD3, SLPI and elafin mRNA, but not HBD4 (Figure 4.1 A-F).

Natural antimicrobial mRNA expression was significantly different from that in FL and WISH cell lines. HBD2 mRNA was expressed in primary amnion cells but undetectable in FL and WISH cells (Figure 4.1 B) and HBD1 and HBD3 were expressed at higher levels in primary amnion cells than in FL and WISH cells (Figure 4.1 A and C; $P<0.05$). Conversely, SLPI and elafin mRNA were significantly lower in primary amnion cells than in either FL or WISH cells (Figure 4.1 E and F; $P<0.05$). Defensin mRNA expression in FL and WISH cells was similar to that seen in the He-La cervical cell line that contaminates them, but SLPI and elafin mRNA expression was lower in FL cells than in He-La cells (Figure 4.1 E and F; $P<0.05$).

4.3.2 Expression of natural antimicrobial mRNA in amnion, choriodecidua and placenta.

Amnion tissue expressed mRNA for HBD1, HBD2 (2/3 samples), HBD3, SLPI and elafin, but not HBD4 (Table 4.1). HBD1, HBD3, SLPI and elafin were also expressed in the choriodecidua and placenta, whereas HBD2 was undetectable in these tissues. There were no significant differences in levels of expression of the other natural antimicrobials between the three tissue types, although there was a trend for increased expression of HBD3 in the amnion.

	HBD1	**HBD2**	**HBD3**	**HBD4**	**SLPI**	**Elafin**
Amnion	**0.20** (0.07)	**0.38** (0.20)	**0.62** (0.33)	**0**	**1.15** (0.43)	**0.1** (0.07)
Chorio-decidua	**0.95** (0.75)	**0**	**0.004** (0.002)	**0**	**1.96** (1.04)	**0.26** (0.11)
Placenta	**0.17** (0.04)	**0**	**0.004** (0.004)	**0**	**0.46** (0.37)	**0.18** (0.06)

Table 4.1

Mean (+/-SEM) expression of natural antimicrobial mRNA in amnion, choriodecidua and placenta obtained at term prelabour Caesarean section at term (n=3), relative to positive control (primary amnion mRNA), as determined by Taqman qPCR.

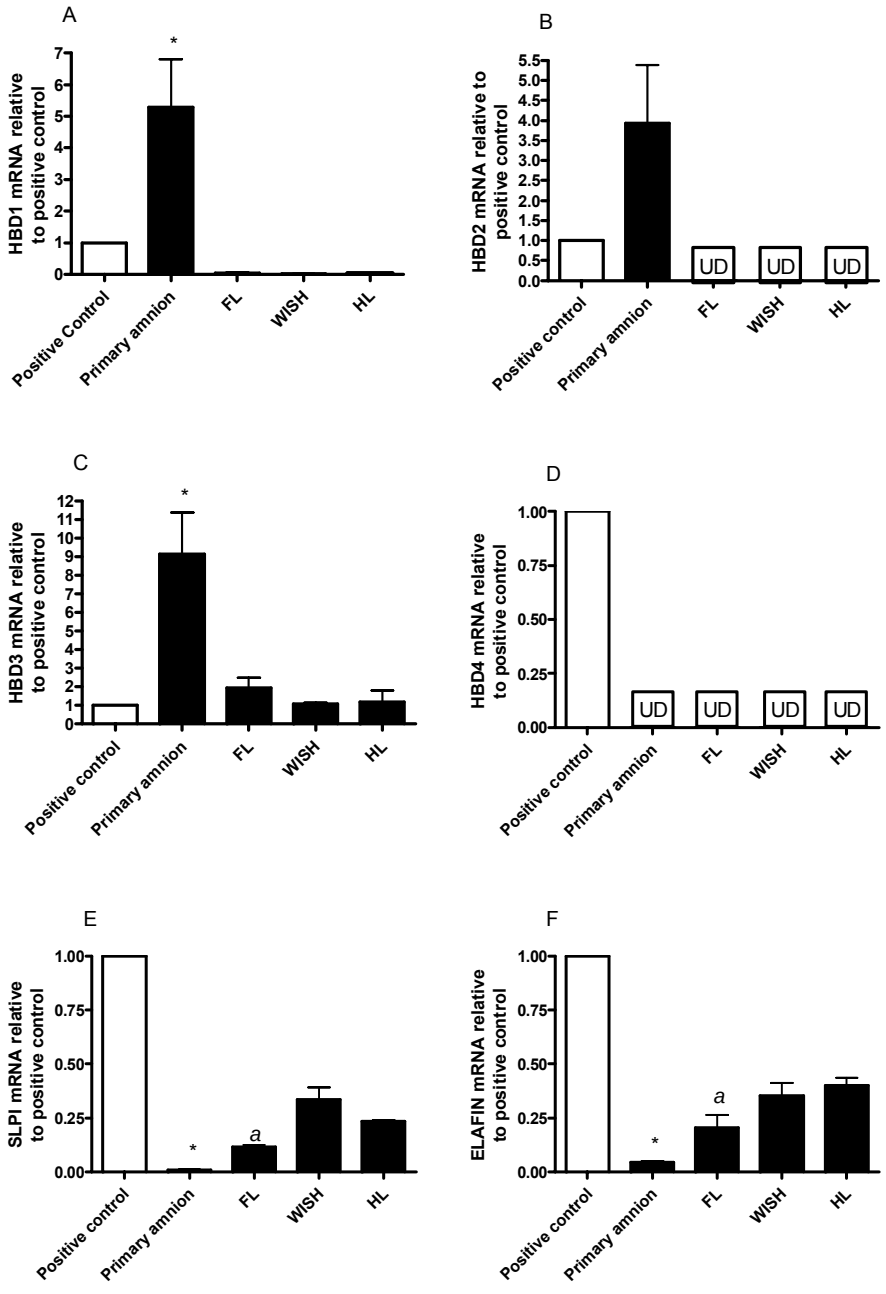


Figure 4.1

Mean +/- SEM expression of natural antimicrobial mRNA in primary cultured amnion cells obtained at prelabour Caesarean section at term (n=5), FL cells (n=3), WISH cells (n=3) and He-La cells (n=3) relative to amount in positive control (endometrial mRNA), as determined by Taqman qPCR. A=HBD1, B=HBD2, C=HBD3, D=HBD4, E=SLPI, F=ELAFIN. UD= undetectable. *P<0.05 primary amnion cells, compared to FL cells, WISH cells and He-La cells. *a*=P<0.05 FL cells compared to He-La cells (One-way ANOVA with Tukey's post-test).

4.3.3 HBD2 mRNA expression was increased in amnion tissue that had been exposed to labour

HBD2 mRNA expression was significantly higher after normal labour (spontaneous vertex delivery) than after prelabour elective Caesarean section (Figure 4.2 B; $P<0.05$). Expression of HBD1, HBD3, SLPI and elafin was not significantly different in labour or non-labour settings (Figure 4.2 A, C, D, E).

4.3.4 IL-1β increased HBD2 expression in amnion tissue explants

Treatment with IL-1β 10ng/ml for 24 hours upregulated expression of HBD2 mRNA (Figure 4.3 A; $P<0.05$), and protein (Figure 4.3 B; $P<0.05$) in amnion explants but had no significant effect on the production of HBD1, HBD3, SLPI or elafin (data not shown).

4.3.5 IL-1β increased HBD1 and HBD2 expression in primary cultured amnion cells

Treatment with IL-1β 10ng/ml upregulated expression of HBD1 and HBD2 mRNA in primary cultured amnion cells at 6 and 12 hours (Figure 4.4 A; $P<0.05$ and B; $P<0.001$), but had no significant effect on the production of HBD3, SLPI or elafin (Figure 4.4.A, C, D and E).

4.3.6 IL-1β had a dose response effect on HBD2 mRNA expression in primary cultured amnion epithelial cells

IL-1β had a dose responsive effect on HBD2 mRNA, which peaked at 1ng/ml and then plateaued (Fig 4.5; $P<0.05$).

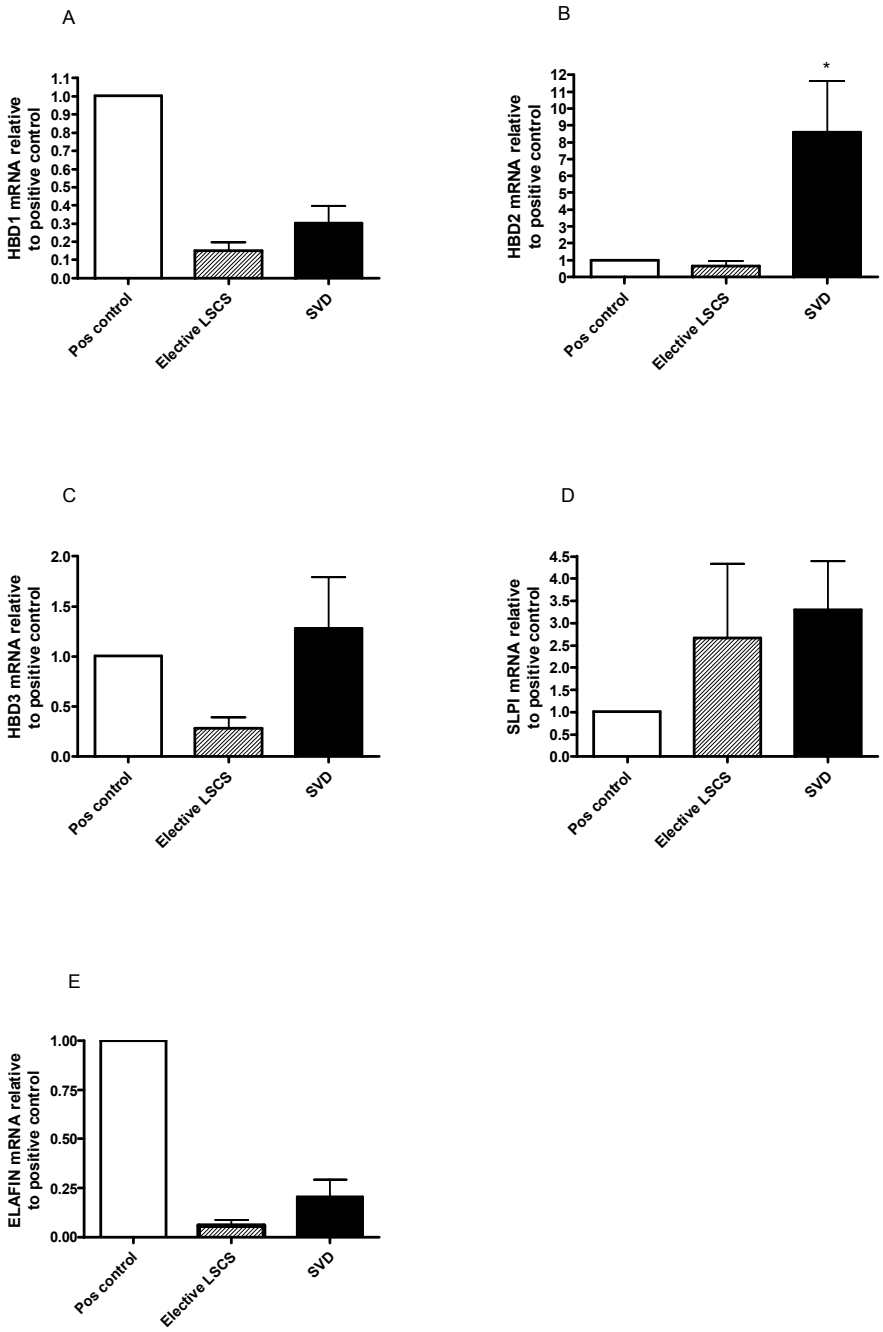


Figure 4.2

Natural antimicrobial mRNA expression in amnion tissue obtained at prelabour Caesarean section (n=8) or following normal labour (n=7), relative to positive control (primary amnion mRNA) as determined by Taqman qPCR. A=HBD1, B=HBD2, C=HBD3, D=SLPI, E=ELAFIN. * $P<0.05$ (Unpaired t-test El LSCS vs SVD).

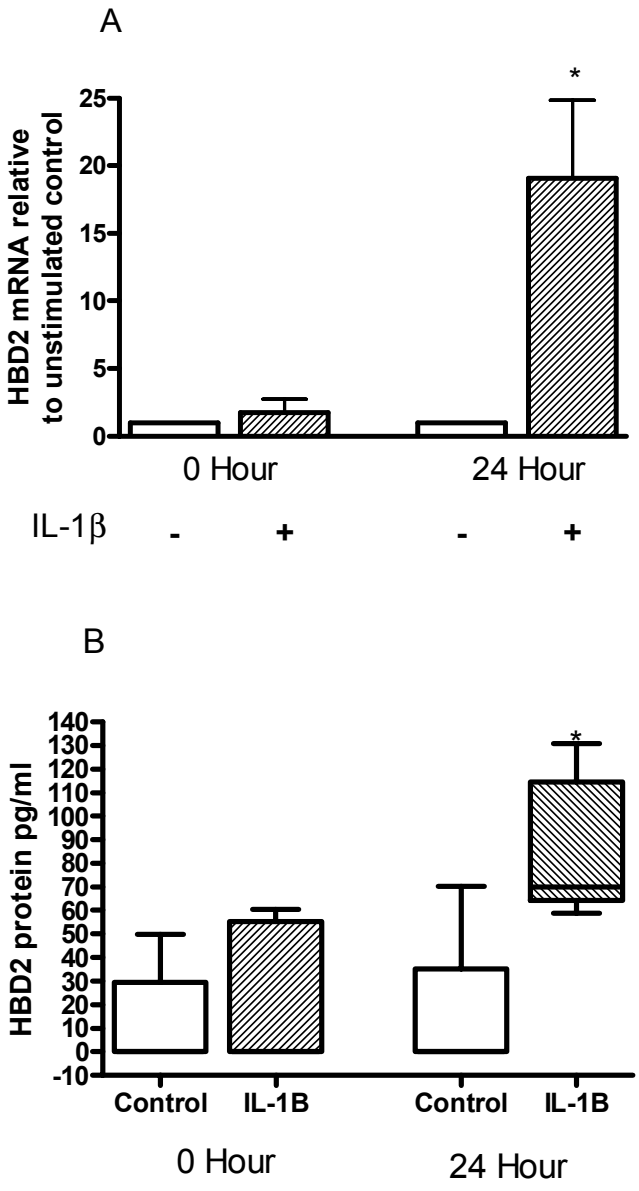


Figure 4.3

A Mean +/- SEM expression of HBD2 mRNA in amnion explants obtained at prelabour Caesarean section at term (n=4) and treated with IL-1β 10ng/ml for 0 and 24 hours, relative to the amount in time matched unstimulated control, as determined by Taqman qPCR. *P<0.05 (One-way repeated measures ANOVA with Tukey's post-test). B Median +/- interquartile range amount of HBD2 (pg/ml) secreted by amnion explants obtained at prelabour Caesarean section at term (n=5) +/- IL-1β 10ng/ml treatment at 0 and 24 hours, as measured by ELISA. *P<0.05 (Friedman test).

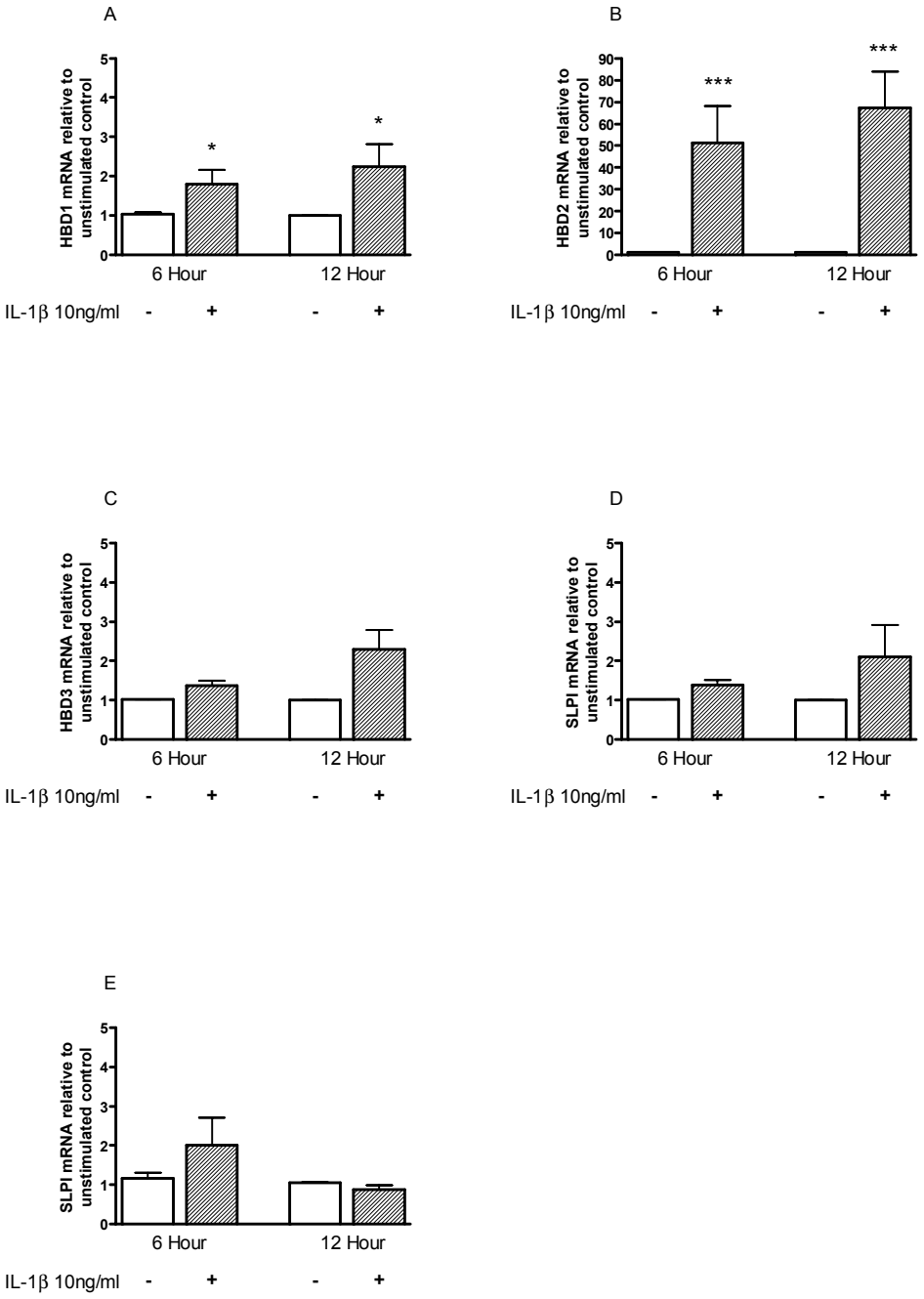


Figure 4.4

Mean +/- SEM expression of natural antimicrobial mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=5) and treated with IL-1β 10ng/ml for 6 and 12 hours, relative to amount in time matched unstimulated control, as determined by Taqman qPCR. A=HBD1, B=HBD2, C=HBD3, D=SLPI, E=ELAFIN. *P<0.05 ***P<0.001 (One–way repeated measures ANOVA with Tukey's post-test).

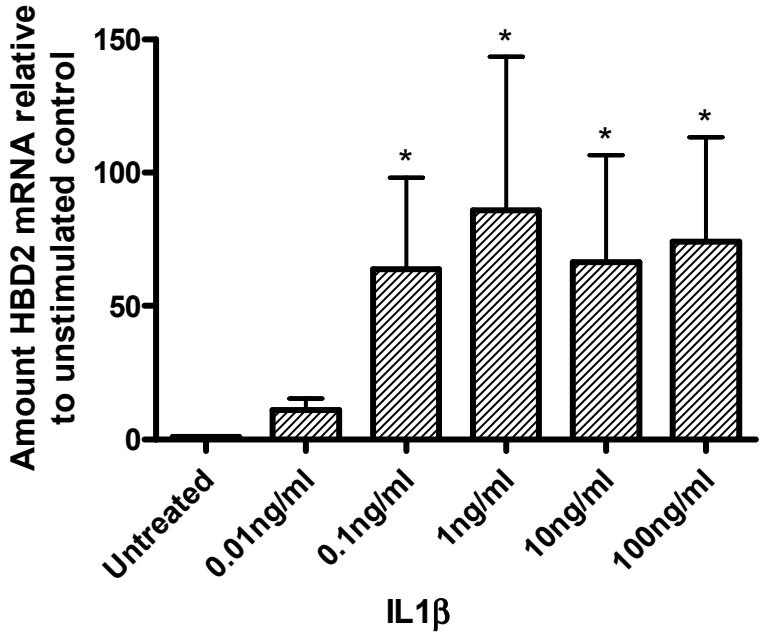


Figure 4.5

Mean +/- SEM expression of HBD2 mRNA in primary cultured amnion cells (n=3) after 6 hours of treatment with IL-1β 0.01ng/ml, 0.1ng/ml, 10ng/ml or 100ng/ml, relative to amount in untreated control, as determined by Taqman qPCR. * $P<0.05$ (One-way repeated measures ANOVA with Tukey's post-test).

4.3.7 IL-1β had a time dependent effect on HBD2 expression in primary cultured amnion epithelial cells

The early response of HBD2 to IL-1β treatment was investigated at 1, 2, 3 and 6 hours in four samples. This showed that HBD2 mRNA expression became significantly upregulated after 3 hours of treatment (Fig 4.6 A; $P<0.05$).

The pattern of HBD2 mRNA expression in response to more sustained treatment with IL-1β was examined in five biological samples at 3, 6, 12, 16, 24 and 48 hours. Treatment significantly upregulated HBD2 mRNA production compared to unstimulated control at all time points examined (Fig 4.6 B; $P<0.05$). There was no significant change in HBD2 mRNA expression in unstimulated controls over the 48 hour period (Fig 4.6 C).

In all five samples HBD2 mRNA was produced in a biphasic pattern (Fig 4.6 D) with two peaks in expression. A typical response from one sample is shown, as the periodicity of the response varied between samples, thus the pattern was not as evident when data were averaged.

HBD2 protein production was significantly increased by treatment with IL-1β 10ng/ml at 24 and 48 hours (Figure 4.7; $P<0.001$).

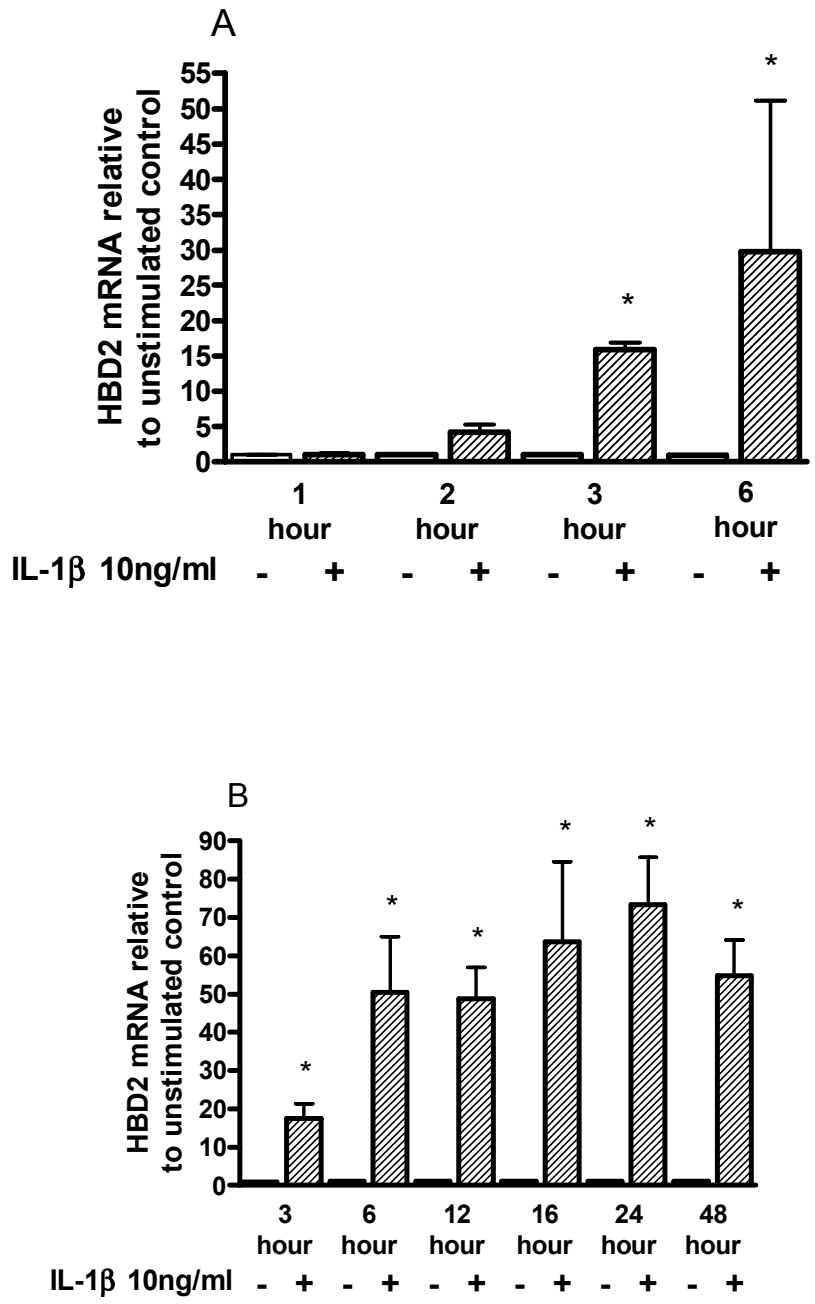


Figure 4.6 A and B

Mean +/- SEM expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (A: n=4; B: n=5) and treated with IL-1β 10ng/ml for 1, 2, 3 and 6 hours (A) or 3, 6, 12, 16, 24 and 48 hours (B), relative to amount in time matched unstimulated control, as determined by Taqman qPCR.*P<0.05 (One-way repeated measures ANOVA with Tukey's post-test).

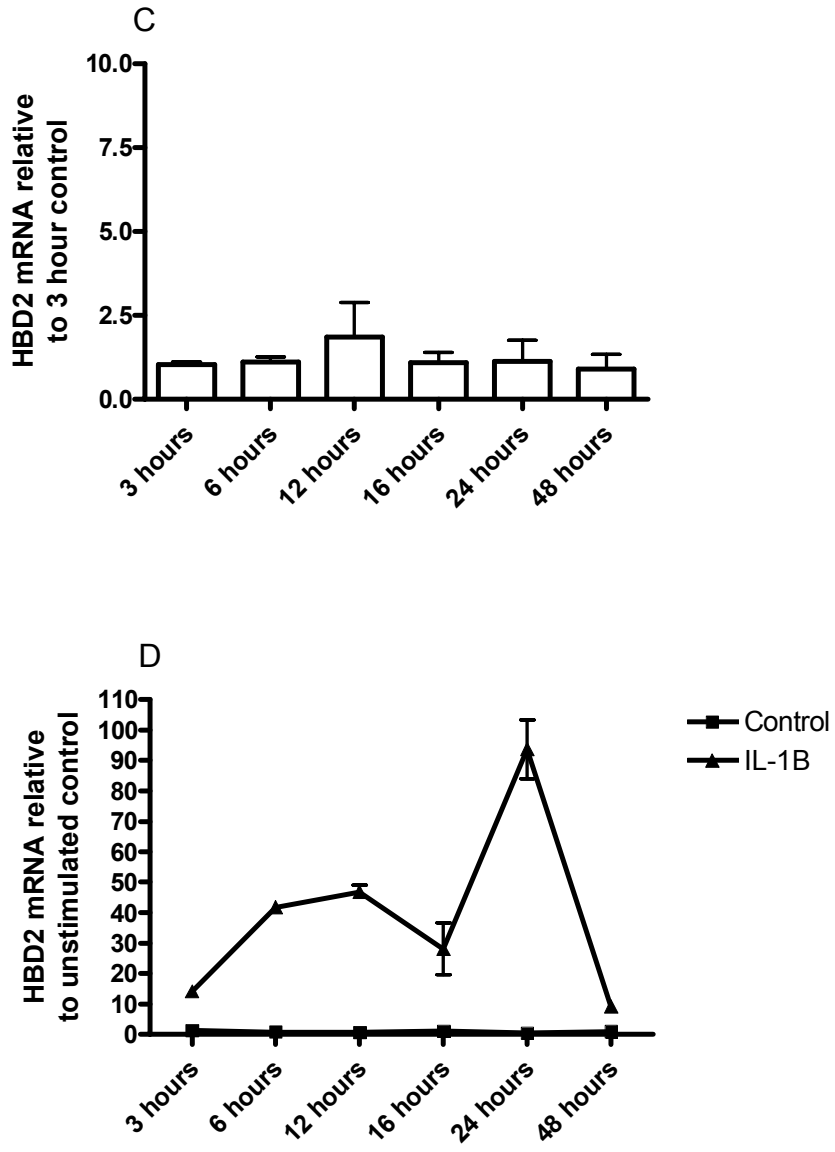


Figure 4.6 C and D

C: Mean +/- SEM expression of HBD2 mRNA over 48 hours, in untreated primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=5), relative to the amount in the 3 hour sample, as determined by Taqman qPCR.

D: Mean expression of HBD2 mRNA in a typical sample of primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=1), +/- IL-1β 10ng/ml at 3, 6, 12, 16, 24 and 48 hours relative to amount in time matched unstimulated control, as determined by Taqman qPCR.

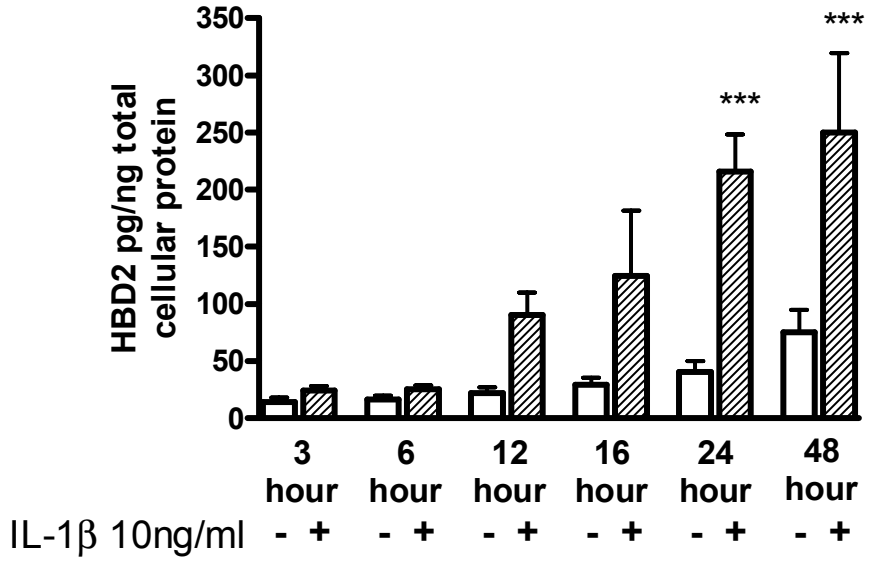


Figure 4.7

Mean +/- SEM amount of HBD2 (pg/ng total cellular protein) secreted by primary cultured amnion cells obtained at prelabour Caesarean section at term (n=5) +/- treatment with IL-1β 10ng/ml at 3, 6, 12, 16, 24 and 48 hours, as measured by ELISA. ***P<0.001 (One-way repeated measures ANOVA with Tukey's post-test).

4.3.8 The effects of TNFα and LPS on HBD2 expression in primary cultured amnion cells were different from those of IL-1β

4.3.8.1 *TNFα*

Treatment with TNFα 10ng/ml upregulated expression of HBD2 mRNA in primary cultured amnion cells at 6 and 12 hours (Figure 4.8 A; $P<0.001$), but had no significant effect on the production of HBD1, HBD3, SLPI or elafin (data not shown).

When the temporal response of HBD2 to TNFα stimulation was compared to that of IL-1β, the patterns of expression were markedly different. HBD2 expression was significantly lower at each time point in response to TNFα (Figure 4.8 B; $P<0.05$).

4.3.8.2 *LPS*

Treatment with LPS 1μg/ml for 6 and 24 hours had no significant effect on expression of HBD1, HBD2, HBD3, SLPI or elafin mRNA (Data not shown).

4.3.9 The effect of IL-17 on HBD2 expression in primary cultured amnion epithelial cells was similar to that of IL-1β

Treatment with IL-17 10ng/ml upregulated expression of HBD2 mRNA in primary cultured amnion cells at 6 and 12 hours (Figure 4.9 A; $P<0.001$), but had no significant effect on the production of HBD1, HBD3, SLPI or elafin (data not shown).

The pattern of HBD2 expression in response to IL-17 was very similar to that of IL-1β, and there was no significant difference in mRNA expression in response to either cytokine (Figure 4.9 B and C).

In contrast, IL-17 and IL-1β invoked distinct patterns of mRNA expression of another innate immune effector, IL-8 (Figure 4.9 D).

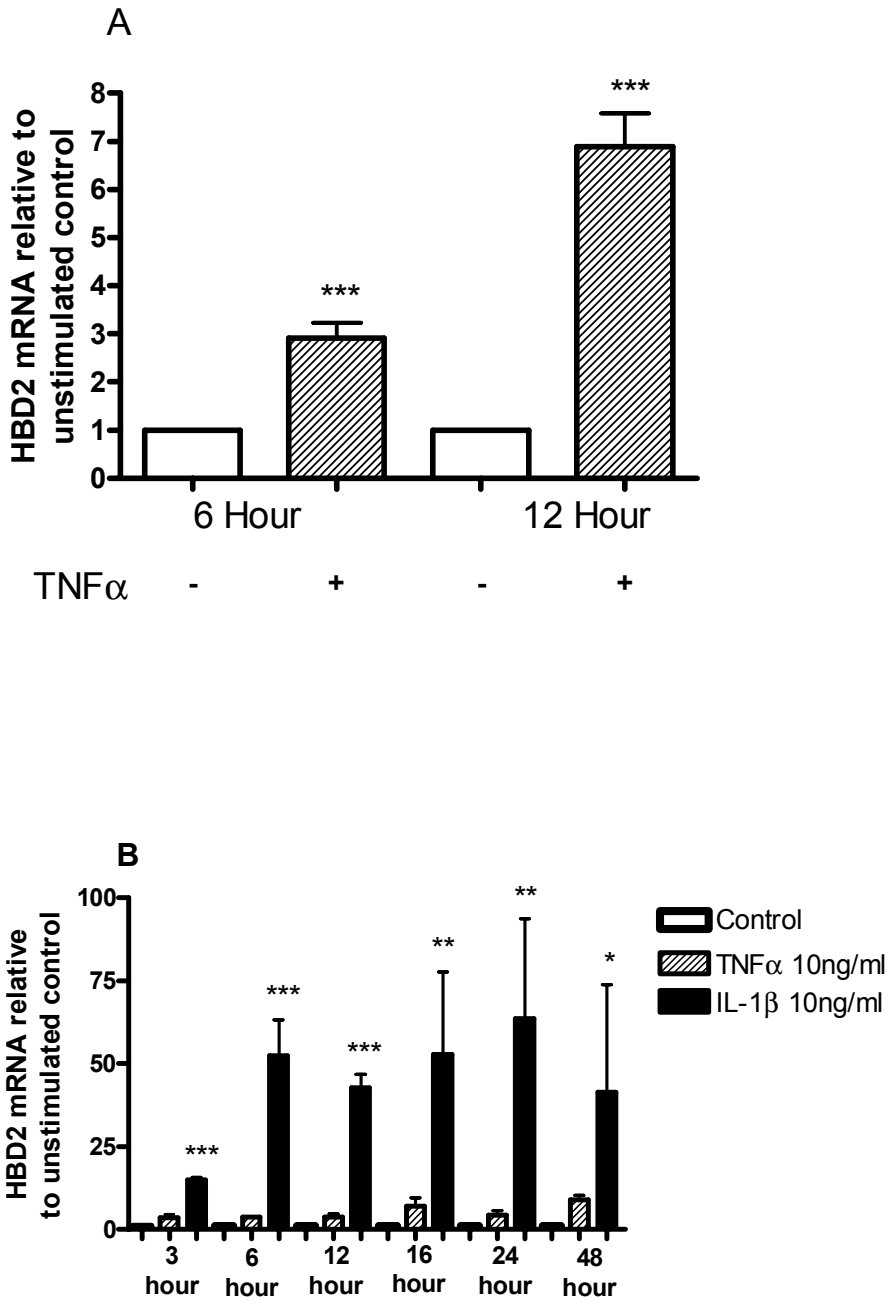


Figure 4.8

Expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term, relative to amount in time matched unstimulated control, as determined by Taqman qPCR. A. Mean +/- SEM after treatment with TNFα10ng/ml for 6 and 12 hours (n=3) B. Mean +/- SEM after treatment with TNFα 10ng/ml for 3, 6, 12, 16, 24 and 48 hours (n=2). *P<0.05 **P<0.01***P<0.001 (One –way repeated measures ANOVA with Tukey's post-test)

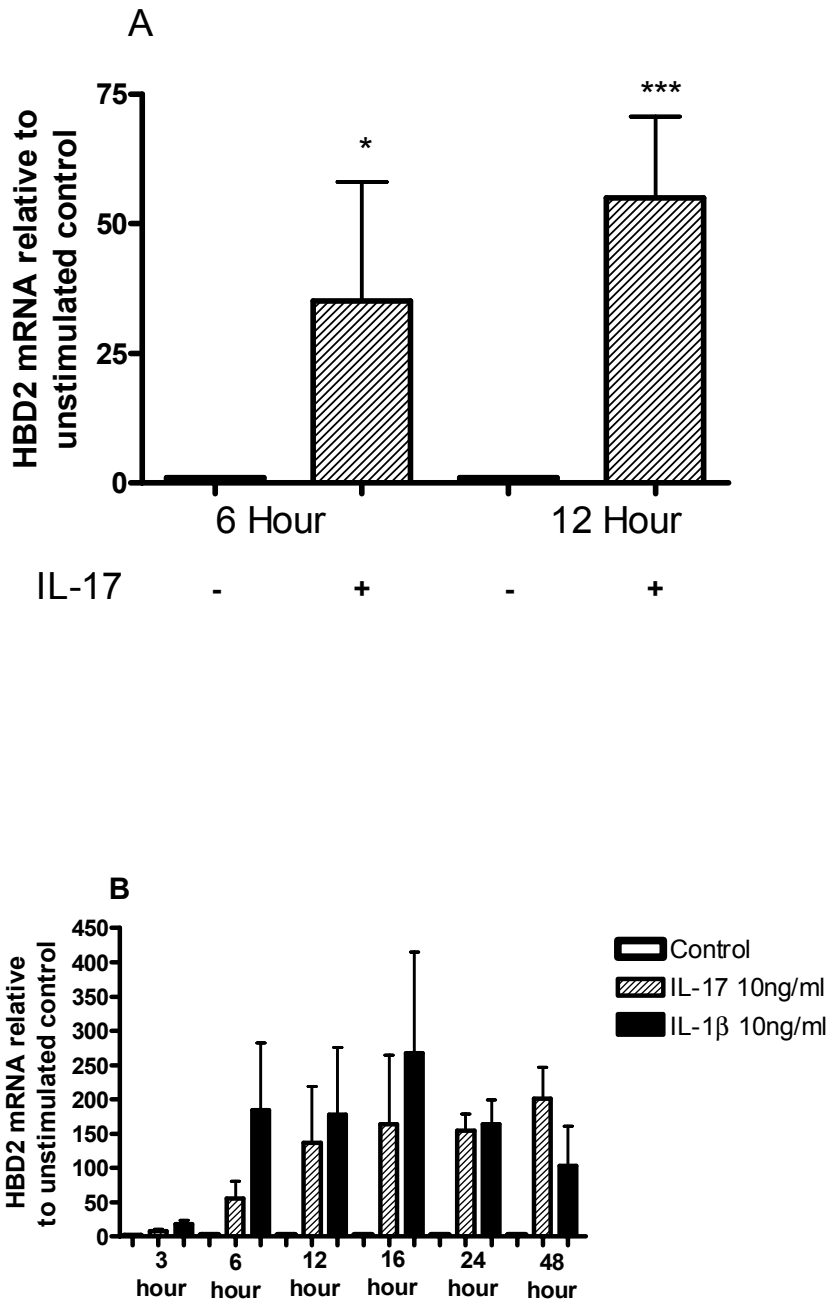


Figure 4.9 A and B

Expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term, relative to amount in time matched unstimulated control, as determined by Taqman qPCR. A. Mean +/- SEM after treatment with IL-17 10ng/ml for 6 and 12 hours (n=3) B. Mean +/- SEM after treatment with IL-17 10ng/ml for 3, 6, 12, 16, 24 and 48 hours (n=4). *P<0.05 ***P<0.001 (One–way repeated measures ANOVA with Tukey's post-test).

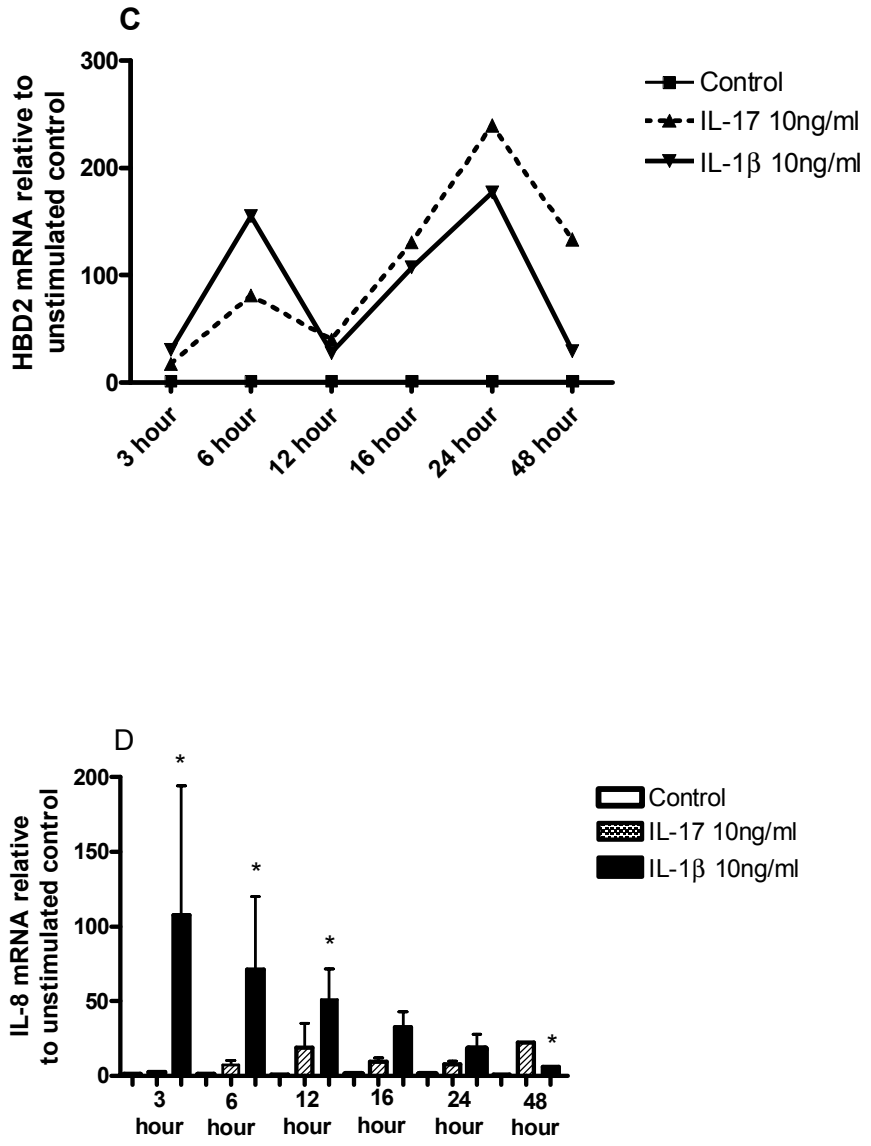


Figure 4.9 C and D

C. Typical pattern of expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term, relative to amount in time matched unstimulated control, as determined by Taqman qPCR after treatment with IL-17 10ng/ml or IL-1β 10ng/ml for 3, 6, 12, 16, 24 and 48 hours (n=1).

D. Mean +/- SEM IL-8 mRNA expression in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term, relative to amount in time matched unstimulated control, as determined by Taqman qPCR after treatment with IL-17 10ng/ml or IL-1β 10 ng/ml for 3, 6, 12, 16, 24 and 48 hours (n=3). *$P<0.05$ (One–way repeated measures ANOVA with Tukey's post-test).

4.3.10 Dexamethasone abrogates the effect of IL-1β on HBD2 and IL-8 expression in primary cultured amnion epithelial cells

Treatment with dexamethasone 1μM alone had no effect on HBD2 mRNA expression in primary cultured amnion epithelial cells. However, when cells were treated with dexamethasone in addition to IL-1β, HBD2 mRNA expression was significantly less than in cells treated with IL-1β alone at 6 and 24 hours (Figure 4.10 A). The effect of IL-1β on IL-8 mRNA expression was also diminished at 6 hours when dexamethasone was added (Figure 4.10 B).

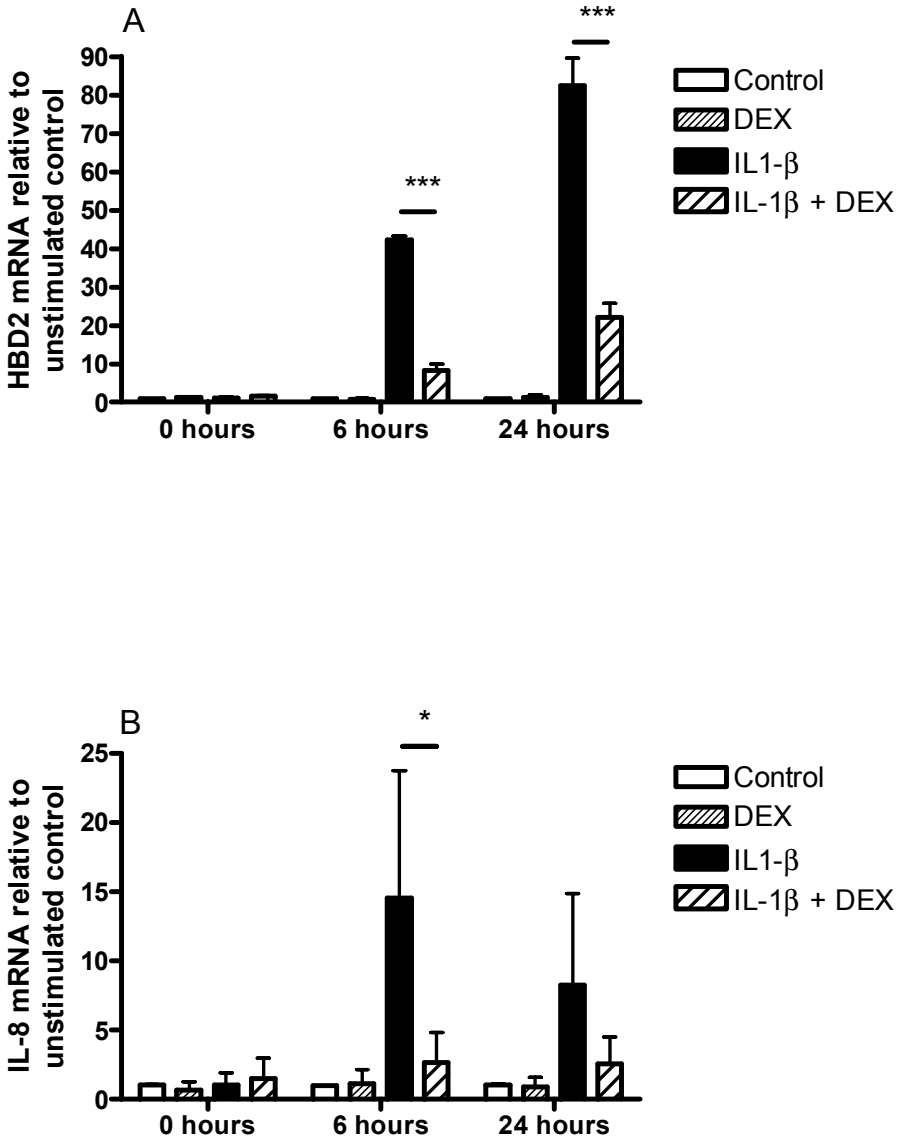


Figure 4.10

Expression of HBD2 (A) and IL-8 (B) mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term, relative to amount in time matched unstimulated control, as determined by Taqman qPCR. Mean +/- SEM after treatment with Dexamethasone 1 μM and/or IL-1β 10ng/ml for 0, 6, and 24 hours (n=2). *P<0.05 ***P<0.001 (One–way repeated measures ANOVA with Tukey's post-test).

4.4 DISCUSSION

Natural antimicrobials are host defence proteins with antibiotic, chemotactic and inflammatory activities. In this chapter their expression in the amnion was examined. Two factors suggested the amnion was an important site to study. Firstly, as it is the innermost fetal membrane, the amnion is critically positioned to protect the fetus from ascending infection. Secondly, the inflammatory response of the amnion is crucial in parturition, as cytokine release from inflammatory cells stimulates amniotic production of prostaglandins. These ripen the cervix and augment myometrial contractions. Amniotic natural antimicrobials could be an important component of the inflammatory response associated with labour.

This study found that the amnion produces five natural antimicrobials. Amnion tissue expresses mRNA for HBD1-3, SLPI and elafin, but not HBD4. It is likely that this is produced by the amnion epithelial cell layer, as primary amnion epithelial cells also expressed mRNA for HBD1-3, SLPI and elafin. Other recent reports have also shown HBD1-3, elafin and SLPI immunopositivity in the amniotic epithelial layer (Zhang, Shimoya et al. 2001; Buhimschi, Jabr et al. 2004; King, Paltoo et al. 2006), and SLPI and elafin mRNA expression in amnion tissue (Zhang, Shimoya et al. 2001; Tromp, Kuivaniemi et al. 2004). It is possible, however, that contaminating cells contributed to production. Some fibroblast contamination is inevitable, but cultures used were >95% positive for the epithelial cell marker pancytokeratin, preliminary experiments showed cultures were consistently >95% negative for the mesothelial cell marker vimentin. The stromal layer of the amnion does contain very few tissue macrophages, which are mainly of fetal origin (Bulmer and Johnson 1984), however it seems unlikely that these cells would persist in cell culture in consistently high numbers to significantly effect results.

Natural antimicrobials produced by the amnion may be secreted into the amniotic fluid, or diffuse through the fetal membranes. Alternatively, as they are highly basic proteins, they may remain associated with the membrane, forming an antimicrobial barrier on the surface of the tissue. Acid washing of endometrial explants has been

shown to increase detachment of natural antimicrobials, for measurement by ELISA (King, Critchley et al. 2000). This technique was tried in preliminary experiments and showed no obvious effect. It may, however, be worth further investigation, especially if used in conjunction with new imaging techniques such as confocal microscopy of amnion explants (Ackerman, Hughes et al. 2005) and the improved antibodies now available for natural antimicrobial study.

Natural antimicrobial expression in commercial cell lines

Only one study has examined defensin production in the amnion before (Buhimschi, Jabr et al. 2004). This concluded that the bacterial wall product LPS, could stimulate expression of HBD3. However it was performed on the so-called, amnion derived FL cell line. Although this was originally created from immortalized primary amnion epithelial cells, it became contaminated with, and eventually replaced by, He-La cells (Nelson-Rees and Flandermeyer 1976; Ogura, Yoshinouchi et al. 1993) (www.atcc.org). The other commercial "amnion" cell line, WISH cells, is similarly affected (Kniss, Xie et al. 2002), nevertheless both these cell lines are frequently used in pregnancy research (Dutheil, Malhomme et al. 1997; Spaziani, Tsibris et al. 1997; Hansen, Drew et al. 1999; Allport, Slater et al. 2000; Keelan, Helliwell et al. 2001; Ackerman, Rovin et al. 2004; Buhimschi, Jabr et al. 2004) and have been cited as having some comparable responses to primary cultured amnion cells (Ackerman, Zhang et al. 2005). When the expression of natural antimicrobials in primary amnion cells was compared to that in FL and WISH cells marked differences were seen. There was significantly greater expression of HBD1, 2 and 3 and lesser expression of SLPI and elafin in primary amnion epithelial cultures than in either cell line. This shows that FL and WISH cells are not valid for the *in vitro* study of amniotic natural antimicrobials. The observation that LPS had no effect on defensin production in primary amnion cells, in contrast to Buhimischi's findings in FL cells (Buhimschi, Jabr et al. 2004), corroborates this. Natural antimicrobial expression in WISH cells did resemble that of He-La cells, although FL cells expressed slightly less SLPI and elafin than He-La cells. This implies there may be phenotypic differences in these

two cell types, despite their similar genetic fingerprint (Nelson-Rees and Flandermeyer 1976).

Natural antimicrobial expression in the fetal membranes

Natural antimicrobial expression was compared in amnion, choriodecidua and placenta. HBD1, HBD3, SLPI and elafin were expressed in all tissue types. HBD2 mRNA was only detectable in the amnion (2/3 samples) and HBD3 expression also tended to be greater in the amnion than in choriodecidua and placenta. Although these results didn't attain significance in the small numbers of samples examined, they did indicate that the amnion provides an important contribution to natural antimicrobial production. The observation that HBD2 mRNA was undetectable in the choriodecidua and placenta contrasts with the findings of King et al, who identified HBD2 in both the chorion trophoblast and syncitiotrophoblast layer of the placenta using immunohistochemistry (King, Paltoo et al. 2006). HBD2 is classically thought of as an inducible natural antimicrobial and expression is usually negligible in the absence of inflammatory stimuli. Indeed, when King et al examined cultured placental trophoblast and syncitiotrophoblast, they found HBD2 mRNA was induced by IL-1β. Lack of such stimulation in the prelabour state may explain why it was undetectable in the samples examined in this study.

Expression of HBD2 mRNA was significantly higher in amnion obtained after normal labour, a process mediated by leukocyte invasion of the uterine tissues and increased production of cytokines (Keelan, Blumenstein et al. 2003). Increased HBD2 may be desirable during parturition, as the open cervix provides an easy route for the ascension of infective organisms. HBD2 is also chemotactic for neutrophils (Niyonsaba, Ogawa et al. 2004) and immature dendritic cells and T cells (Yang, Chertov et al. 1999), and thus could participate in the recruitment of inflammatory cells seen in labour. Labour had no effect on production of other natural antimicrobials, suggesting their expression in the amnion at term is either constitutive, or already maximally stimulated before the onset of labour.

IL-1β stimulation of natural antimicrobials

IL-1β is an inflammatory cytokine which stimulates natural antimicrobial production in a variety of tissues (Singh, Jia et al. 1998; Mathews, Jia et al. 1999; O'Neil, Porter et al. 1999; Garcia, Krause et al. 2001; Harder, Bartels et al. 2001; King, Fleming et al. 2002). Levels of IL-1β are increased in the fetal membranes and amniotic fluid at parturition (Keelan, Blumenstein et al. 2003), and IL-1β can stimulate labour in animal models (Sadowsky, Adams et al. 2006). These factors suggested that IL-1β could be involved in upregulation of HBD2 in the amnion seen after normal labour. Its effect on natural antimicrobial production in amnion explants was thus examined. IL-1β resulted in a significant increase in HBD2 expression, at both mRNA and protein level, but did not affect other natural antimicrobials. This was consistent with the findings from tissues exposed to labour, suggesting IL-1β may be responsible for the effect.

Amnion consists of a layer of epithelial cells with an underlying layer of sparsely distributed fibroblasts, either of which could be responsible for the production of natural antimicrobials observed in tissue explants. However, primary amnion epithelial cells cultured in isolation responded to treatment with IL-1β in a similar way to full thickness explants. This indicates that HBD2 from the amnion is an epithelial product, and probably little modulation by fibroblasts occurs.

IL-1β also resulted in an increase in expression of HBD1 mRNA which was statistically significant. Until recently, it was widely accepted that HBD1 was constitutively produced by epithelia whereas HBD2 was highly inducible by various cytokines, microbes and bacterial products (Krisanaprakornkit, Weinberg et al. 1998; Harder, Meyer-Hoffert et al. 2000; Hao, Zhao et al. 2001; Nitschke, Wiehl et al. 2002; McDermott, Redfern et al. 2003). Recent studies, however, have demonstrated HBD1-4 may all be produced in a constitutive or inducible manner, depending on the site of production and stimulus applied (Yang, Biragyn et al. 2004; Joly, Organ et al. 2005; Vankeerberghen, Nuytten et al. 2005). Nevertheless, HBD1 expression was not increased after normal labour, and the increase in HBD1 mRNA observed in response to IL-1β in the amnion was only marginal; thus it may not be clinically

relevant. A lack of suitable antibodies meant that it was not possible to verify an increase of HBD1 protein.

IL-1β did not influence primary amnion epithelial mRNA expression of SLPI. This differs from findings by Zhang et al, who demonstrated an increase in SLPI protein production by primary amnion cells after stimulation with IL-1β (Zhang, Shimoya et al. 2001).The effect was only seen after 48 hours, however, which is not typical of an innate immune response, which is characteristically rapid. It may represent a secondary response, not seen at the time points examined in this study.

Elafin mRNA expression is increased in samples of whole fetal membranes in cases of chorioamnionitis (Tromp, Kuivaniemi et al. 2004), and IL-1β treatment stimulates elafin production in chorion trophoblast cells (King, Paltoo et al. 2006). In contrast, IL-1β had no effect on elafin expression in the amnion. This could suggest that chorion provides the greater contribution to elafin production in infected fetal membranes.

HBD2 expression in the amnion was further investigated in primary amnion epithelial cells, to characterize production. IL-1β increased HBD2 mRNA expression in a dose responsive way, which peaked at 1ng/ml and then plateaued. Subsequent timecourse experiments used a dose of 10ng/ml, to allow for any possible metabolism of the molecule. When examined over 48 hours, IL-1β upregulated both HBD2 mRNA and protein. The pattern of HBD2 mRNA production in response to IL-1β was intriguing, and has not been described before. In all 5 samples examined, HBD2 mRNA showed a biphasic response, though the periodicity of the response varied. The first peak in production occurred within a few hours, which is typical of a rapid innate immune response. The second peak was slower, and may represent stimulation by a secondary gene product. This theory is further investigated in the next chapter.

The effect of other inflammatory mediators

The effects of other stimulants on the amnion were examined, to see if they also produced a biphasic pattern of HBD2 mRNA expression. LPS is a Gram-negative bacterial wall product which activates the innate immune response via Toll-like receptor 4 (TLR4) (see Section 1.2.6.5). This receptor shares the same cytoplasmic domain as the IL1R (Toll/Interleukin Restriction or TIR domain) and can activate identical pathways (Aderem and Ulevitch 2000). However LPS had no effect on HBD2 expression in the amnion, even though expression of TLR4, and its necessary co-factor CD14, was confirmed by Taqman PCR (data not shown). This contrasts with findings in epidermal keratinocytes (Harder, Bartels et al. 1997), oral mucosa (Mathews, Jia et al. 1999) and endometrium (King, Fleming et al. 2002), where both IL-1β and LPS influence HBD2 production, suggesting that different mucosa have developed distinct regulatory mechanisms of natural antimicrobial production.

Several factors can reduce susceptibility to LPS, which could operate in the amnion. These include expression of negative regulators of the NFκB pathway such as TOLLIP (toll interacting protein) and IRAK-M (IL-1 receptor associated kinase), and expression of a non-signalling form of MYD88 (for review see (Cario and Podolsky 2005). Availability of the receptor may be another factor modulating the LPS response as the amnion exhibits trafficking of TLR4 from the apical surface to the basal surface of cells in the presence of infection (Adams, Lucas et al. 2006). It is of interest that intestinal epithelium also demonstrates shuttling of TLR4 in the presence of infection (Cario, Brown et al. 2002), and IL-1β, but not LPS, can stimulate HBD2 production in colonic epithelial cell lines (O'Neil, Porter et al. 1999), resembling the response in the amnion.

Even if LPS does not directly stimulate HBD2 production from amnion epithelial cells, it may indirectly influence production by provoking IL-1β release from other cells such as neutrophils and macrophages. In this situation it could act to limit the spread of infection, and may also augment neutrophil chemotaxis (Figure 4.11). The expression of natural antimicrobials in cases of preterm labour and/or infection is an area for subsequent investigation.

TNFα and IL-17 are two other key cytokines of the innate immune response. Although IL-1β, TNFα and IL-17 signal through distinct receptors, they utilize the same signalling pathways – NFκB and MAPK, thus their effect on HBD2 production was examined.

HBD2 was stimulated by TNFα treatment in a markedly different pattern to IL-1β. On the other hand IL-17 and IL-1β had very similar effects on HBD2, although different effects on another innate immune effector, IL-8. Taken together these findings suggest that inflammatory signalling in the amnion is regulated at more than one level, with influences occurring downstream to the ligand /receptor interaction. This is a characteristic of the innate immune system, which is dependent on only a few pathways, but can generate a variety of effects due to multiple regulatory mechanisms, at both transcriptional and post-transcriptional levels (Kracht and Saklatvala 2002). Putative factors in the control of the HBD2 response in the amnion are investigated in the next chapter.

TNFα is upregulated in amniotic fluid and fetal membranes in association with labour (Romero, Manogue et al. 1989; Laham, Brennecke et al. 1994; Stallmach, Hebisch et al. 1995; Arntzen, Kjollesdal et al. 1998; Weiyuan and Li 1998; Shobokshi and Shaarawy 2002), but less is known about IL-17 expression in pregnancy. It is produced mainly by activated CD4+ T-cells, as well as neutrophils and eosinophils (Kawaguchi, Adachi et al. 2004). It has not been conclusively identified in murine trophoblast (Chaouat, Zourbas et al. 2002; Ostojic, Dubanchet et al. 2003), but has recently been identified in human trophoblast from term placentas, early miscarriage and molar pregnancy, as well as Hofbauer cells (Pongcharoen, Somran et al. 2007). There are no published reports if IL-17, or its receptor, in the fetal membranes. This study showed that amnion cells could respond to IL-17 *in vitro*. Further investigations should be performed to establish whether it has a role *in vivo.* In particular, its involvement in the process of parturition should be assessed, as IL-17 can induce PTGS2 (COX2) expression in keratinocytes (Kanda, Koike et al. 2005) and intestinal myofibroblasts (Zhang, Andoh et al. 2005).

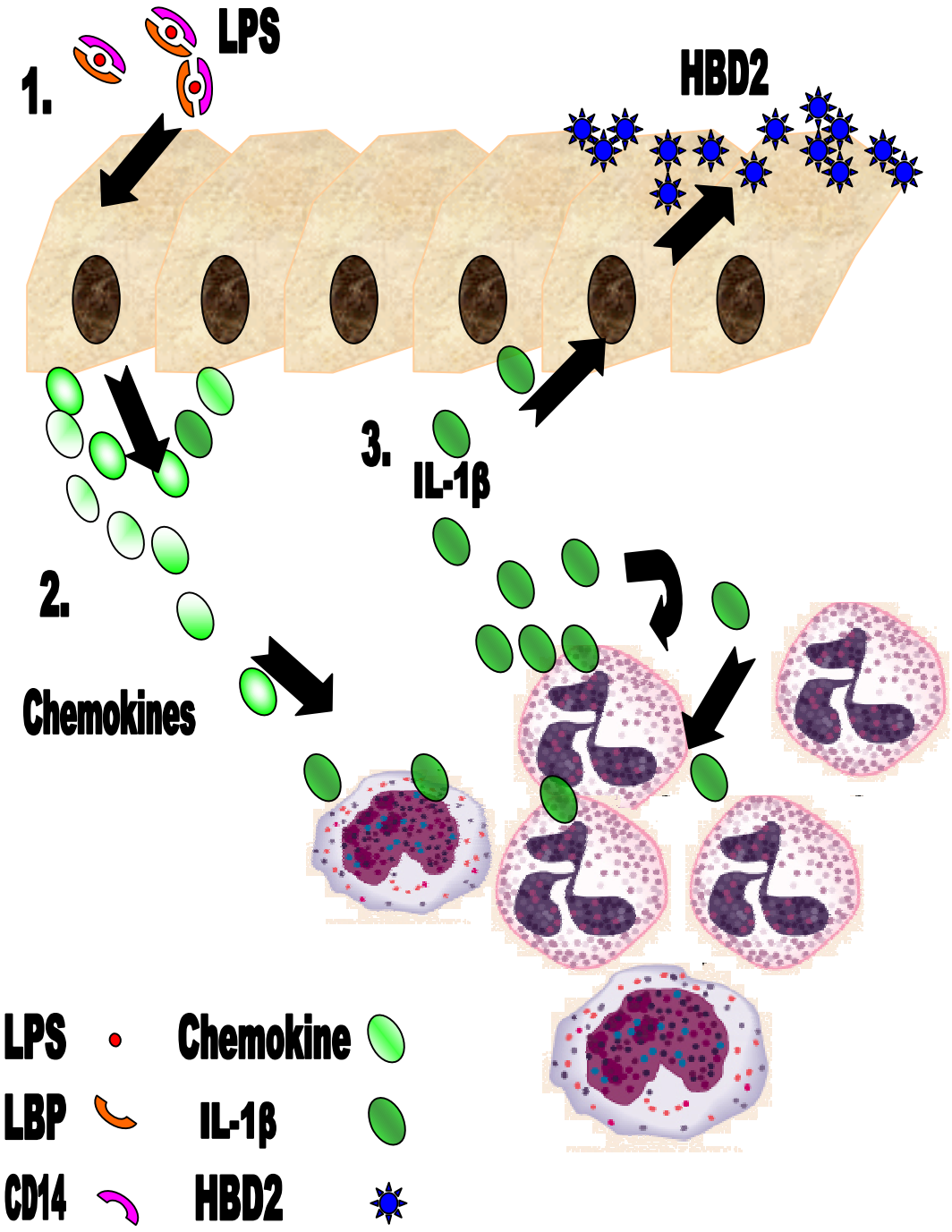


Figure 4.11

Stimulation of HBD2 in amnion epithelial cells

1. LPS, in conjunction with LBP and CD14, activates amnion epithelial cells; 2. Chemokines are released attracting neutrophils and macrophages; 3. IL-1β is released from inflammatory cells, which promotes further chemotaxis; 4. IL-1β stimulates

production of HBD2, which forms a protective barrier on the amnion surface, and augments chemotaxis of inflammatory cells

Dexamethasone upregulates HBD2 in intestinal cell lines (Witthoft, Pilz et al. 2005), and has some pro-inflammatory functions in the amnion, such as the upregulation of prostaglandin E2 and PTGH-2 in amnion fibroblasts (Whittle, Gibb et al. 2000; Sun, Ma et al. 2003). It had no effect on HBD2 expression in the amnion when applied in isolation. Nevertheless, it did diminish the effect of IL-1β on both HBD2 and IL-8 production, perhaps via its ability to inhibit both NFκB and AP-1 DNA binding (Lukiw, Pelaez et al. 1998). The involvement of these signalling pathways are further explored in the next chapter.

Although glucocorticoids are commonly used in clinical obstetrics to promote fetal lung maturation in threatened preterm labour, caution is advised in administration in cases of chorioamnionitis due to a theoretical risk they may worsen infection (RCOG Green top guideline No 7, RCOG press 2004). The findings of this study support this recommendation, as modification of amniotic HBD2 production by glucocorticoids could increase susceptibility to penetrative infections, with deleterious effects to the fetus.

Summary

In summary, the experiments in this chapter demonstrate expression of HBD1, 2 and 3, and SLPI and elafin in the amnion. IL-1β and IL-17 dramatically upregulate production of HBD2 in the amnion in a dose and time-dependant manner. HBD2 is a potent natural antibiotic, which also interacts with the adaptive immune system. Further study may allow development of new strategies and treatments to help decrease the incidence of premature birth.

5. The effect of IL-1β on the amniotic epithelium

5.1. INTRODUCTION

It was shown in Chapter 4 that when amnion epithelial cells are stimulated by IL-1β, HBD2 mRNA is expressed in a biphasic pattern. The periodicity of this response varies somewhat between samples, but two peaks in production are consistently seen; one occurring at 6 or 12 hours, and a second at 16 or 24 hours. This suggested that one or more secondary gene products may influence HBD2 expression. An unknown protein, also stimulated by IL-1β, may inhibit HBD2 production causing the decrease in expression. Alternatively it could stimulate a second transient rise in its expression. In order to identify factors which could potentially mediate this response, a cDNA microarray was used. As cDNA microarrays provide a means of characterizing gene expression on a genomic scale, they can be more efficient than performing multiple experiments searching for individual factors. Important microarray findings were verified by Taqman qPCR, and mechanisms involved in the response were investigated with pathway inhibitors. Although many results were inconclusive, they will provide direction for further study.

As anticipated the microarray also provided new insights into the inflammatory response of the amnion, which is central to parturition but remains poorly understood. The results are discussed in the context of other recent microarray studies of the amnion, and the potential relevance of the findings is speculated upon.

5.2. METHODS

All materials, reagents and cell lines are detailed in Appendix 1.

5.2.1. Specimen Collection

Samples of amnion were collected as described in Section 2.1.2, from fourteen women undergoing prelabour elective Caesarean section at 39 weeks gestation.

5.2.2. Primary amnion epithelial cell culture and treatments

Primary amnion epithelial cells were cultures as detailed in Section 2.2.1.1.

5.2.2.1. *cDNA microarray and confirmatory PCR*

Cells were treated with recombinant human IL-1β 10ng/ml or equivalent volumes of serum free media for 1, 2, 3, 6 or 8 hours. All treatments were performed in duplicate. RNA was extracted as described in Section 2.3.1 and RNA quality was checked by Agilent Bioanalysis (Section 2.3.2.1).

The RNA replicate with the highest RIN number from one sample was used for microarray analysis. Confirmatory PCR was performed on four separate patient samples as described in Section 2.4. Primer and probe sets are detailed in Section 2.4.3.

5.2.2.2. *IL-8, BMP2, endothelin-1 and indomethacin*

Cells were treated with recombinant human IL-8 10ng/ml; BMP2 10ng/ml; endothelin-1 1nM; or indomethacin 5μM and/or IL-1β 10ng/ml; or equivalent volumes of serum free medium for 0, 6 or 24 hours. The doses chosen were indicated by other published reports: IL-8 (Khabar, Al-Zoghaibi et al. 1997); BMP2 (Kim, Romero et al. 2005) Endothelin-1 (Mitchell, Romero et al. 1990); indomethacin (Sato, Keelan et al. 2002). Treatments were performed in duplicate. RNA was extracted and PCR performed as described in Section 2.3-4.

5.2.2.3. *Pathway inhibitor experiments*

Pathway inhibitors, or equivalent volumes of vehicle, were added as detailed in Table 5.1, with or without IL-1β 10ng/ml for 3, 6, 12, 16, 24 or 48 hours. The doses chosen were shown to be optimal in other published reports (Lappas, Permezel et al. 2002) or in experiments in other cell lines performed by other groups in our laboratory. Treatments were performed in duplicate. RNA was extracted and PCR performed as described in Section 2.3-4.

Pathway	**Inhibitor**	**Dose**	**Vehicle**
NFκB	Sulfasalazine	5μM	Serum free media
ERK	PD96059	50μM	DMSO
JNK	JNKII inhibitor	5μM	DMSO
p38	SB203560	5μM	DMSO
EGF receptor	AG1478	200nM	DMSO

Table 5.1
Pathway inhibitors used in the experiments in this chapter

5.2.3. Oligonucleotide microarray

The Agilent Whole Genome Oligo Microarray platform was used. This contains 44,000 oligonucleotide probes representing 41,000 unique genes and transcripts. These have been verified and optimized by alignment to the human genome assembly and Agilent's empirical validation process. It is a two colour system that allows measurement of the relative expression ratio of a given sample compared to a common reference sample. In this experiment, the reference sample for each array consisted of the pooled control samples, and was labelled with Cy 5 (red). The sample of interest on each microarray was an individual IL-1β treated timepoint and was labelled with Cy 3 (green). In total 5 microarrays were performed.

5.2.3.1. *Amplification*

Amplification of RNA was carried out with Agilent Low RNA Input Fluorescent Amplification kit, to generate fluorescent (Cy3/5) linearly amplified complementary RNA (cRNA). Firstly cDNA was synthesized by incubating 4.25µl of cDNA mastermix (2µl 5x First strand buffer; 1µl 0.1M dithiothreitol [DTT]; 0.5µl 10mM deoxyribonucleoside triphosphates [dNTP] mix; 0.5µl MMLV-reverse transcriptase [MMLV-RT]; 0.25µl RNase out) with 5.75µl nuclease free water containing 50ng total RNA and 0.6µl T7 promoter primer, at 40°C for 2 hours. The MMLV-RT was then heat inactivated by incubation at 65°C for 15 minutes.

cRNA was prepared by adding 1.2µl of Cy 5-CTP to the reference sample (pooled control samples), and 1.2µl of Cy 3-CTP to each of the IL-1β treated samples. These were then incubated with 28.8µl of transcription mastermix (7.65µl Nuclease free water; 10µl transcription buffer; 3µl DTT; 4µl ribonucleoside triphosphate [NTP] mix; 3.2µl 50% polyethylene glycol [PEG]; 0.3µl inorganic pyrophosphatase; and 0.4µl T7 RNA polymerase) at 40°C for 2 hours.

The resulting cRNA was purified using Qiagen RNeasy minispin columns. cRNA was brought up to a volume of 100µl with nuclease free water. 350µl of RLT buffer was added, and mixed with 250µl of ethanol. The resulting mixture was applied to RNeasy mini spin columns and centrifuged for 30 seconds at 8000g, whereupon the lysate was discarded. The columns were transferred to new collection tubes, 500µl of RPE buffer added, and centrifuged for a futher 30 seconds. The lysate was discarded and this step was repeated. cRNA was eluted into a new collection tube, by two centrifugations each with 30µl of nuclease free water. cRNA was stored at -80°C until use for hybridization.

5.2.3.2. *Hybridization*

Hybridization was performed using the Agilent In-situ Hybridization Kit Plus. 0.75µg of Cy-3 and Cy-5 linearly amplified cRNA was added to 50µl of 10x control targets and nuclease free water to give a total volume of 240µl. This was added to 10µl 25x fragmentation buffer, and incubated at 60°C for 30 minutes in the dark.

250µl of 2x hybridization buffer was then added, and the mixture was immediately loaded on the microarray. Microarrays were hybridized for 16 hours at 60°C.

Following hybridization, microarrays were washed for 1 minute each in wash solutions 1 (700 ml nuclease free water; 300ml 20x Saline-sodium-phosphate-EDTA buffer (SSPE); and 0.25ml N-Lauroylsarcosine) and 2 (997ml nuclease free water; 3 ml SSPE; and 0.5ml 20% N-Lauroylsarcosine). They were then immersed for 30 seconds in Stabilization and Drying Solution (from the hybridization kit) which protects against ozone-induced degradation of cyanine dyes on the microarray slides. Slides were stored under nitrogen until scanning.

5.2.3.3. *Scanning, data extraction and quality control*

Microarrays were scanned on an Agilent DNA microarray scanner. Images were analyzed and data extraction performed using Agilent Feature Extraction 8 software.

The relative expression of probes was expressed as the log2 ratio of fluorescence. It is microarray convention to have the green channel as the denominator, meaning it was calculated by "log2 (Red/Green)"; thus in this experiment "log2 (Pooled control/IL-β treated sample)". This means if expression in the treated sample is higher than that of the control, it was represented by a *negative* log2 ratio. A log2ratio of 0 means there was no difference in expression between the treated sample and the pooled control; a value of -1 means that expression in the treated sample was twice that of the pooled control; and a value of -2 means the expression in the treated sample was 4 times (2^{-2}) higher than in the pooled control sample and so on.

Plotting colour-coded background intensity values by their location on the array showed largely uniform background noise. Gradient patterns were only visible at low levels (Appendix 4, Figure A4.1). Expression log2 ratios exhibited no evidence of irregularities on the array (Appendix 4, Figure A4.2).

Boxplots showing the distribution of expression values per dye channel showed generally lower background noise for the green dye-label channel, and good similarity of data distributions for signal intensities (Figure 5.1). The only exception was the sample for the 6 hour time point, which had fewer probes at the high end of expression in both the reference and time point samples.

MA plots (Figure 5.2) show dependencies of expression log ratios on the signal level. The X-axis represents the average expression level for both samples on the array. The Y-axis is the corresponding log2 ratio of expression. Most of the arrays showed a classic “hockey stick” pattern, with globally very different expression ratios at low signal levels. The six hour timepoint again showed some irregularities in the shape of the data cloud.

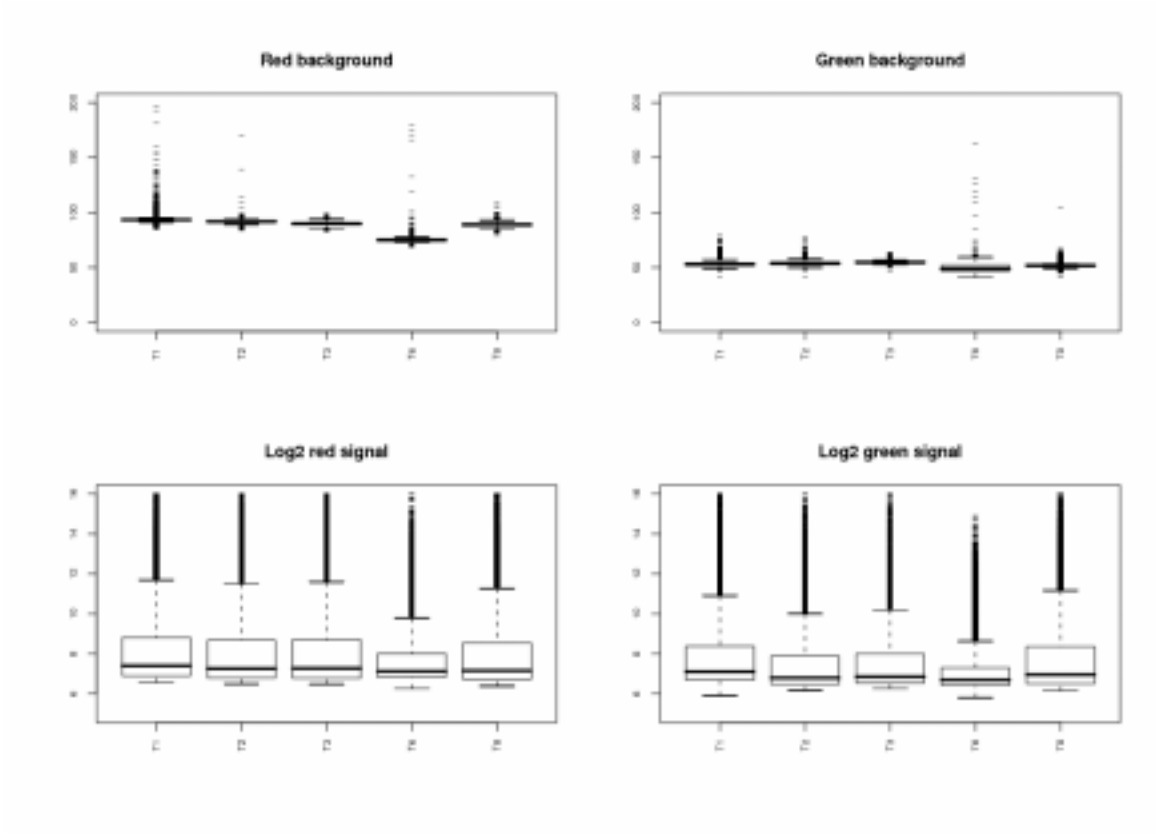


Figure 5.1

Boxplots of expression values (pre-normalisation) per dye-label channel. T1= 1 hour timepoint; T2= 2 hour timepooint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

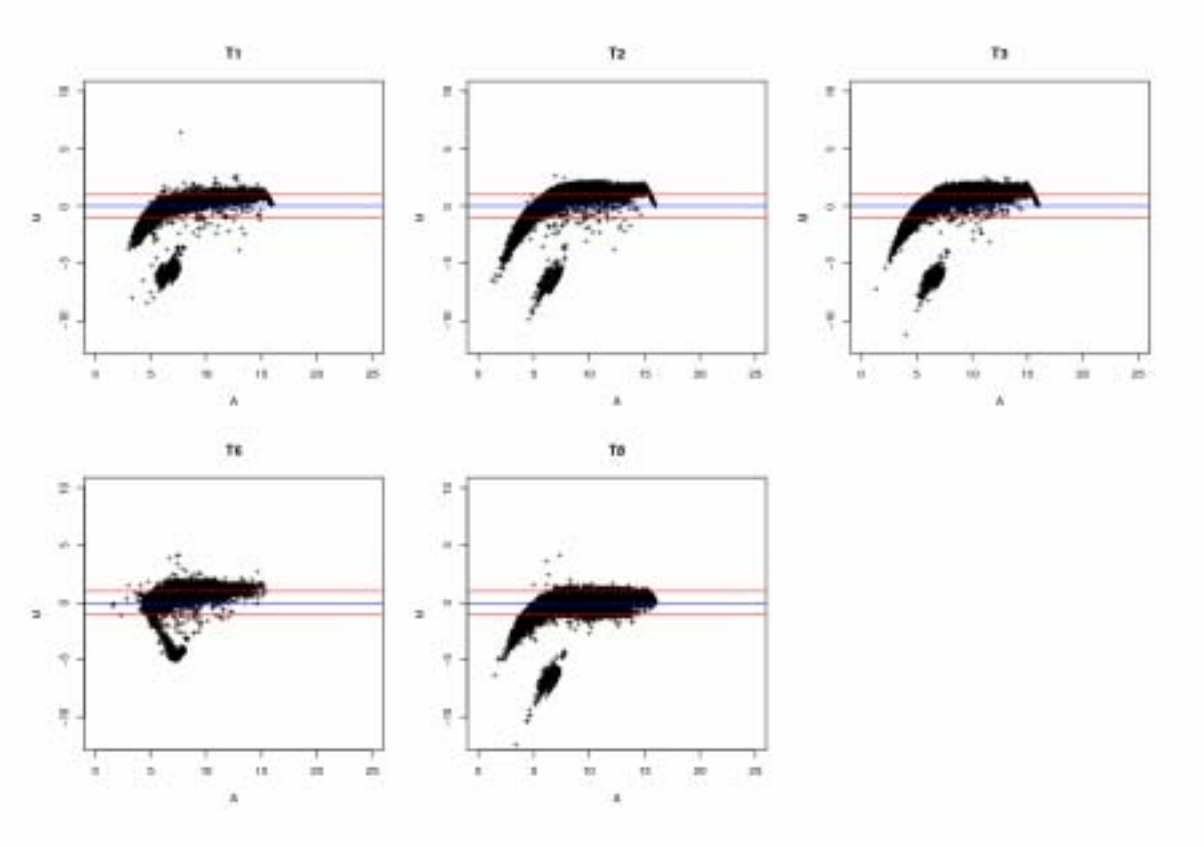


Figure 5.2

MA Plots of pre-normalised data. The X-axis represents the average expression level for both samples on the array. The Y-axis is the corresponding log2 ratio of expression. The blue line represents no fold difference in expression between the two samples. The two red lines represent two-fold up or down regulation. The separate data cloud represents the control probes. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

5.2.3.4. *Normalisation of data*

Edward's correction algorithm was used for background correction, to remove noise from the measured signal values without introducing negative expression values.

LOESS normalisation is a standard technique in dual-dye arrays (Forster, Roy et al. 2003) and was performed on each slide. It is based on the assumption that greater than 90% of probes on the array should show no difference between the reference and time-point sample, thus centres log2 ratios around 0 for all levels of expression. Positive control probes were excluded in the fitting of this model, in order to prevent skewing.

Between-slide normalisation was also performed to allow comparison of relative expression from array to array. This is based on the assumption that the majority of arrays should have a similar range of log2 expression values.

Repeat MA plots were created of the normalized data for quality control (Figure 5.3), which as expected show that the log2 expression ratios are now centred around the no-differentiation line (y=0) for the entire range of expression levels. Separate MA plots of positive, negative and bright corner spots were performed, to determine these behaved as expected (Figure 5.4).

Boxplots for log2 ratio distributions were matched between arrays (Figure 5.5). Only the 6 hour timepoint differed, with fewer upregulated probes in the timepoint samples (negative values). RNA from this timepoint was re-labelled and hybridised, but these differences persisted. Some caution must therefore be used in interpreting these results.

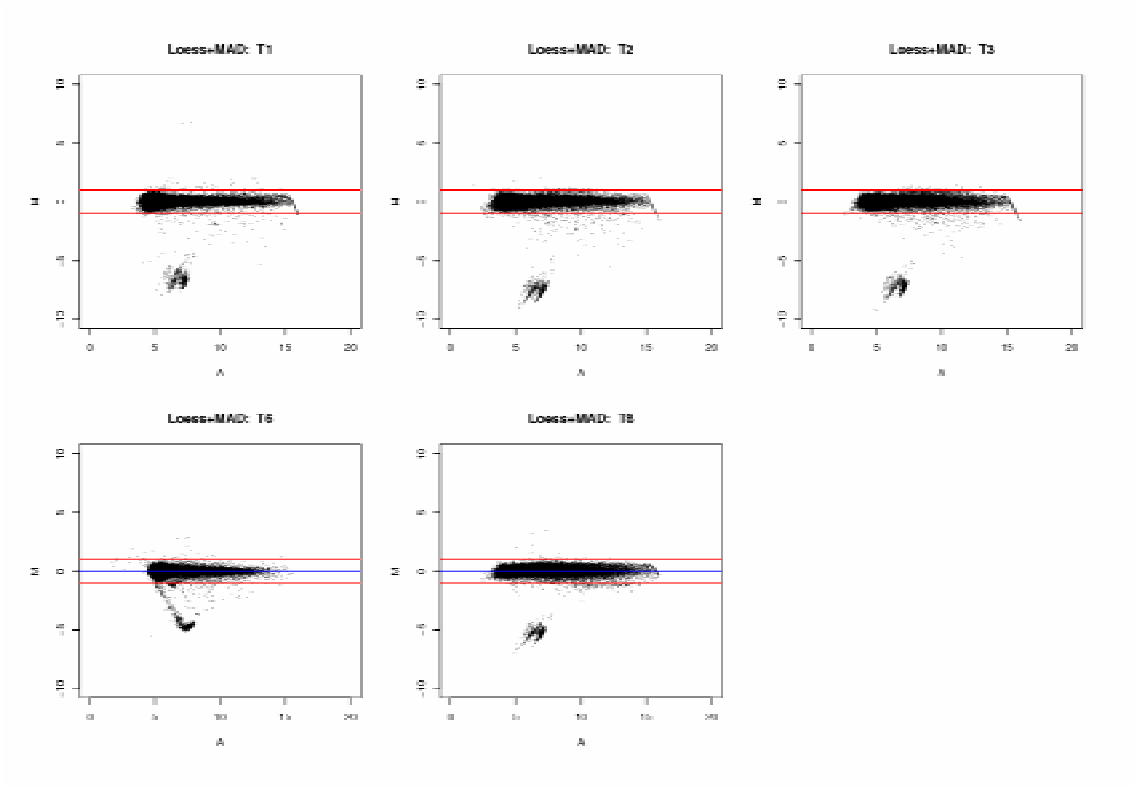


Figure 5.3

MA Plots of normalised data (without control probes). The X-axis represents the average expression level for both samples on the array. The Y-axis is the corresponding log2 ratio of expression. The blue line represents no fold difference in expression between the two samples. The two red lines represent two-fold up or down regulation. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

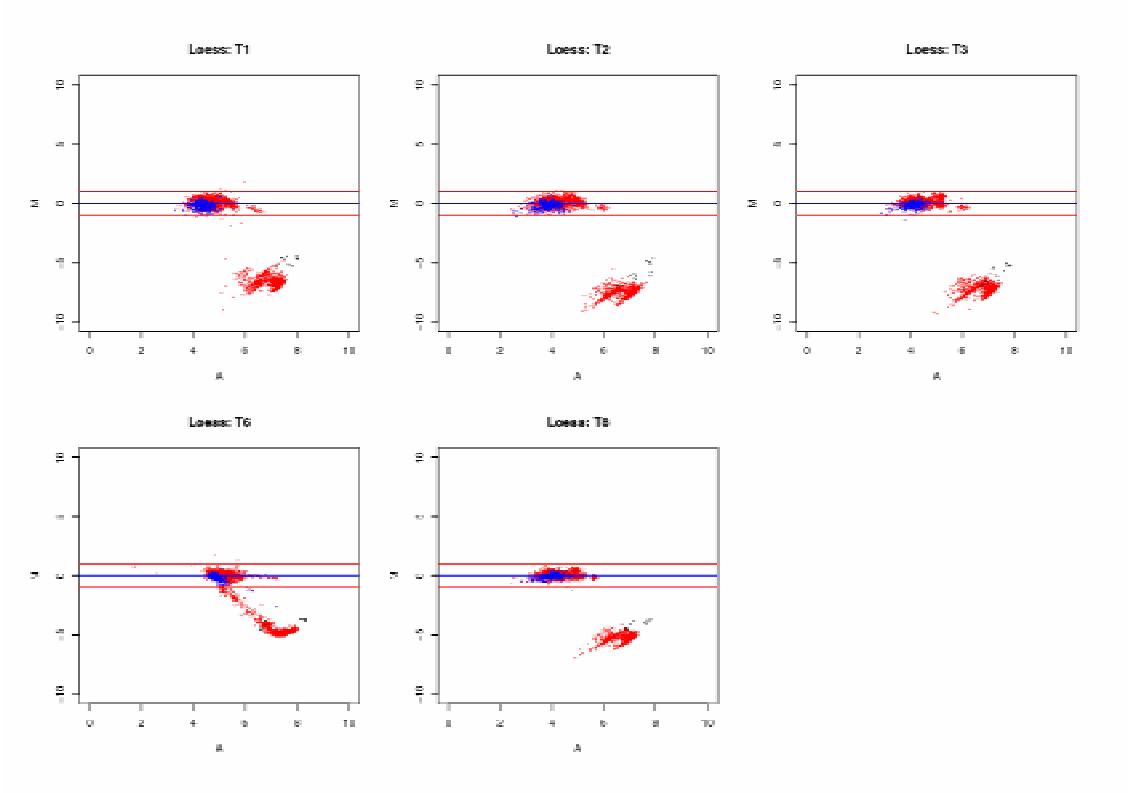


Figure 5.4

MA Plots of controls. The X-axis represents the average expression level for both samples on the array. The Y-axis is the corresponding log2 ratio of expression. The blue line represents no fold difference in expression between the two samples. The two red lines represent two-fold up or down regulation. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

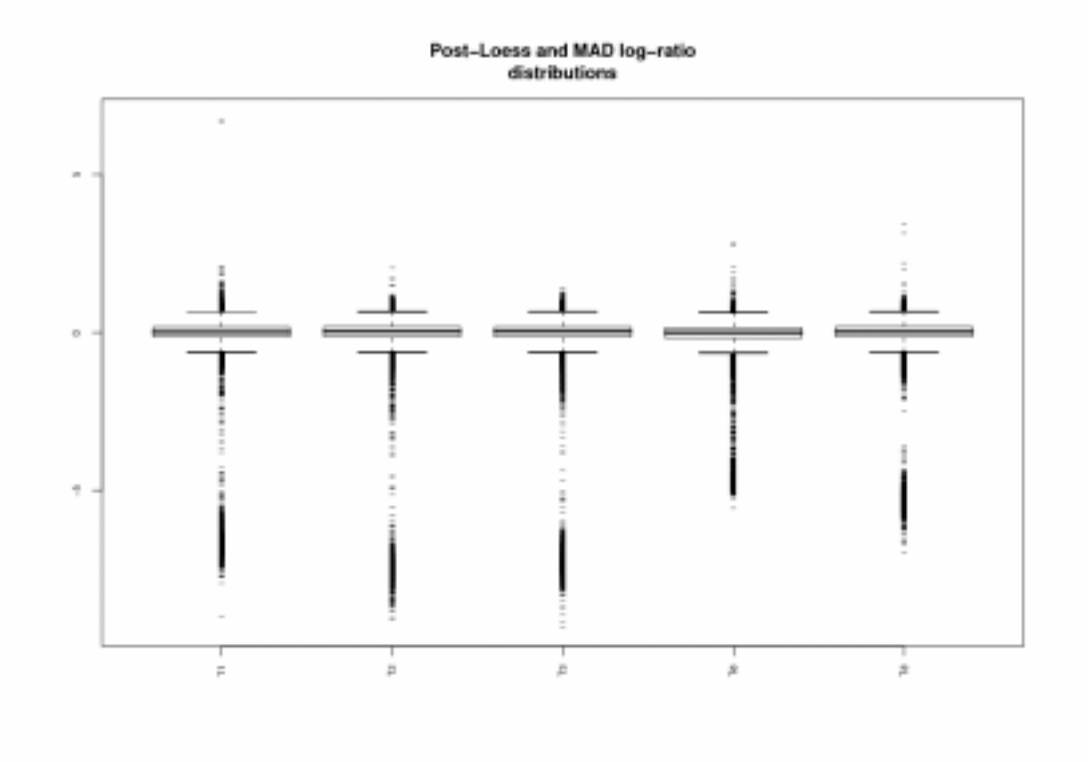


Figure 5.5

Boxplots of expression values of normalised data. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

5.2.4. Confirmatory Taqman qPCR

Reverse transcription and Taqman qPCR were carried out as described in Section 2.3.-4

5.2.5. Data mining and gene ontology analysis

Interrogation of microarray results was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov). This is a web-based application that allows users to access a relational database of functional annotation. Functional annotations are derived primarily from LocusLink at the National Center for Biotechnology Information (NCBI). DAVID uses LocusLink accession numbers to link gene accessioning systems to biological annotations including gene names and aliases, functional summaries, Gene Ontologies, protein domains, and biochemical and signal transduction pathways. Annotation pedigrees are provided via direct links to the primary sources of annotation, which also provide additional gene specific information (Dennis, Sherman et al. 2003).

In order to obtain insight into the functional and clinical significance of genes, gene-ontology analysis was performed. This indicates components involved in particular biological processes which were over-represented compared to chance. These results are not conclusive as the hierarchical structure of gene ontology annotation causes problems with statistical analysis which are as yet unresolved. They do however, provide direction for further investigation.

5.2.6. Amnion Tissue

6 samples of amnion obtained at prelabour Caesarean section, and 5 samples obtained after spontaneous vertex delivery were collected as described in Section 2.1.2. RNA was extracted as detailed in section 2.3, and analysed for CD69

expression by Taqman quantitative PCR, using the method and primer and probe set detailed in Section 2.4.

5.2.7. Statistical Analysis

Statistical analysis on raw microarray data is described above, whilst any further manipulations of the normalized data are described in the appropriate results section. One-way repeated measure ANOVA, with Tukey's post test to assign individual differences, was performed on ΔCT values in the Taqman PCR data.

5.3. RESULTS

5.3.1. Visualizations

Global results (no filtering) are represented in a scatterplot (Figure 5.6). This plots the control sample of each array versus the corresponding time point. Global results are also detailed in Appendix 6, which is on the attached compact disc (CD).

5.3.2. Filtering

Data was filtered by removing all control probes, and any probes that did not have a log2 expression of greater than 1 in at least 1 microarray. This left a total of 1022 probes of interest. These are listed in Appendix 6 on the attached CD.

The majority of genes were upregulated in response to IL-1β, with only 106 repressed genes identified (Appendix 6; CD). Many of these have as yet unassigned functions, and there were no obvious candidates for involvement in the HBD2 response.

5.3.3. K-means clustering

Logarithmically transformed data of the filtered genes was sorted using the K-means algorithm, to generate 16 clusters. This method identified subsets of genes with similar patterns of expression (Figure 5.7). The gene for HBD2 (DEFB4) was located in cluster 4.

Of particular interest were K-means clusters 7 and 15, representing genes that are upregulated at 1, 2 and 3 hours, but decrease again at 6 and 8 hours (Table 5.2). The kinetics of these clusters made it feasible that products of these genes may negatively influence production of HBD2. Several genes were examined by multiple probe sets, which were clustered together, demonstrating the reproducibility of the data.

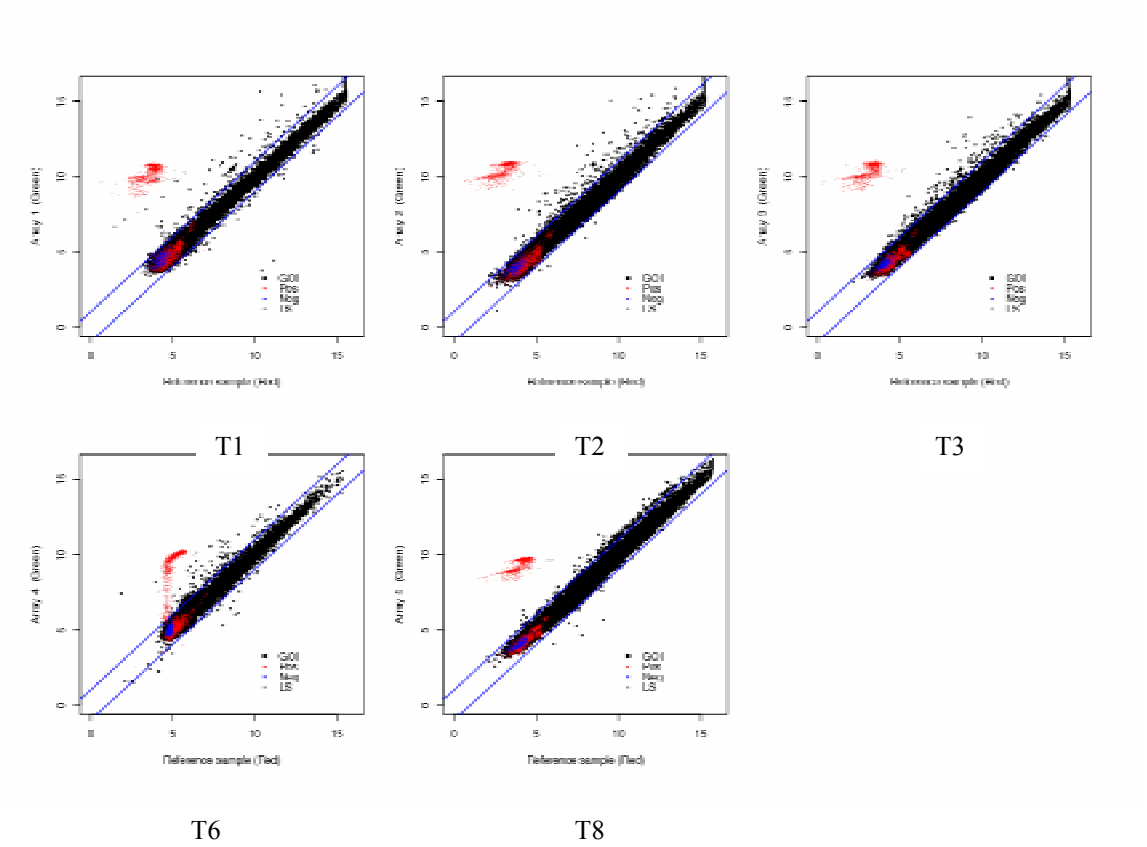


Figure 5.6

Scatterplots of expression profiles representing 1022 genes that have different expression profiles in pooled control sample versus the IL-1β treated sample of primary amnion epithelial cells at each time point. Black dots represent genes of interest, red dots represent positive control probes, blue dots represent negative control probes, and grey dots represent “bright corners” (landing spots). T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

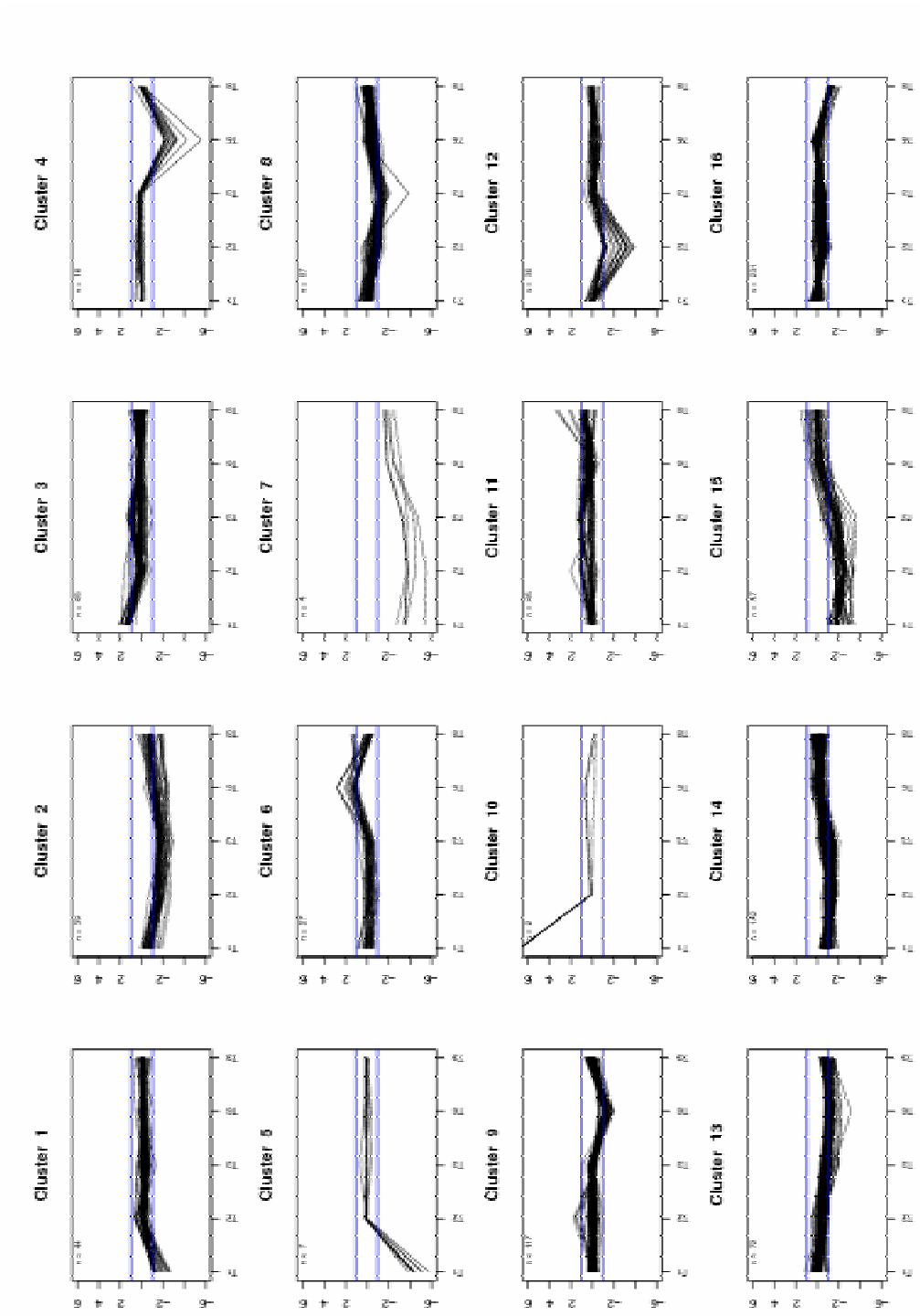


Figure 5.7

K-means clustering of patterns of gene expression. The x axis shows timepoint. The y axis shows l of the reference compared to sample. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

GeneName	SystematicName	Description
IL8	NM_000584	interleukin 8 (IL8)
CCL20	NM_004591	chemokine (C-C motif) ligand 20 (CCL20)
CXCL1	NM_001511	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (CXCL1)
CXCL2	NM_002089	chemokine (C-X-C motif) ligand 2 (CXCL2)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
EFNA1	NM_004428	ephrin-A1 (EFNA1), transcript variant 1
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
KLF6	NM_001300	Kruppel-like factor 6 (KLF6), transcript variant 2
IL1A	NM_000575	interleukin 1, alpha (IL1A)
KLF6	NM_001300	Kruppel-like factor 6 (KLF6), transcript variant 2
NFκBIZ	NM_031419	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFκBIZ), transcript variant 1
PLAU	NM_002658	plasminogen activator, urokinase (PLAU)
EREG	NM_001432	epiregulin (EREG)
ATF3	NM_004024	activating transcription factor 3 (ATF3)
ZFP36	NM_003407	zinc finger protein 36, C3H type, homolog (mouse) (ZFP36)
BMP2	NM_001200	bone morphogenetic protein 2 (BMP2)
MAP3K8	NM_005204	mitogen-activated protein kinase kinase kinase 8 (MAP3K8)
PTGS2	NM_000963	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2)
OVOL1	NM_004561	ovo-like 1(Drosophila) (OVOL1)
CYR61	NM_001554	cysteine-rich, angiogenic inducer, 61 (CYR61)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
DUSP1	NM_004417	dual specificity phosphatase 1 (DUSP1)
TNFAIP3	NM_006290	tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)
TA-NFκBH	NM_032721	T-cell activation NFκB-like protein (TA-NFκBH)
EGR1	NM_001964	early growth response 1 (EGR1)
BC015987	BC015987	clone IMAGE:4096273
PHLDA1	NM_007350	pleckstrin homology-like domain, family A, member 1 (PHLDA1)
EFNA1	NM_004428	ephrin-A1 (EFNA1), transcript variant 1
PTGER4	NM_000958	prostaglandin E receptor 4 (subtype EP4) (PTGER4)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
CYR61	NM_001554	cysteine-rich, angiogenic inducer, 61 (CYR61)
TNFAIP3	NM_006290	tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)
CEBPD	NM_005195	CCAAT/enhancer binding protein (C/EBP), delta (CEBPD)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
NFκBIA	NM_020529	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFκBIA)
TNF	NM_000594	tumor necrosis factor (TNF superfamily, member 2) (TNF)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
CXCL2	NM_002089	chemokine (C-X-C motif) ligand 2 (CXCL2)
IER2	NM_004907	immediate early response 2 (IER2)

EDN1	NM_001955	endothelin 1 (EDN1)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
P2RY4	NM_002565	pyrimidinergic receptor P2Y, G-protein coupled, 4 (P2RY4)
PTGS2	NM_000963	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2)
CD69	NM_001781	CD69 antigen (p60, early T-cell activation antigen) (CD69)
TRIB1	NM_025195	tribbles homolog 1 (Drosophila) (TRIB1)
CXCL3	NM_002090	chemokine (C-X-C motif) ligand 3 (CXCL3)
NR4A1	NM_002135	nuclear receptor subfamily 4, group A, member 1 (NR4A1), transcript variant 1
AK092260	AK092260	cDNA FLJ34941 fis, clone NT2RP7007480
HSPC159	NM_014181	HSPC159 protein (HSPC159)

Table 5.2

Probes forming k-means clusters 7 and 15.

5.3.4. Gene ontology analysis

In order to obtain insight into the functional and clinical significance of the genes in clusters 7 and 15, gene-ontology analysis was performed. This identified 13 "bioprocesses", 7 "molecular functions" and 1 "cellular components" which were over-represented compared to chance (Table 5.3 and Table5.4).

GO Classification	GO ID	GO Name	P value
GO Biological Process	GO:0006954	inflammatory response	2.44E-06
GO Biological Process	GO:0045087	innate immune response	4.37E-06
GO Biological Process	GO:0006935	chemotaxis	4.70E-06
GO Biological Process	GO:0042330	taxis	4.70E-06
GO Biological Process	GO:0009607	response to biotic stimulus	8.67E-06
GO Biological Process	GO:0009605	response to external stimulus	3.79E-05
GO Biological Process	GO:0009611	response to wounding	4.95E-05
GO Biological Process	GO:0009613	response to pest/pathogen/parasite	2.07E-04
GO Biological Process	GO:0006952	defense response	3.16E-04
GO Biological Process	GO:0042221	response to chemical substance	4.53E-04
GO Biological Process	GO:0006955	immune response	9.55E-04
GO Biological Process	GO:0006950	response to stress	3.01E-03
GO Biological Process	GO:0007154	cell communication	1.97E-02
GO Molecular Function	GO:0005125	cytokine activity	5.91E-06
GO Molecular Function	GO:0005102	receptor binding	8.02E-05
GO Molecular Function	GO:0004871	signal transducer activity	7.42E-04
GO Molecular Function	GO:0042379	chemokine receptor binding	1.20E-03
GO Molecular Function	GO:0008009	chemokine activity	1.20E-03
GO Molecular Function	GO:0042056	chemoattractant activity	1.42E-03
GO Molecular Function	GO:0001664	G-protein-coupled receptor binding	1.42E-03
GO Cellular Component	GO:0005615	extracellular space	1.13E-04

Table 5.3

Gene ontology classifications that were statistically over-represented in clusters 7 and 15 on gene-ontology analysis. P was determined by Bonferonni's test.

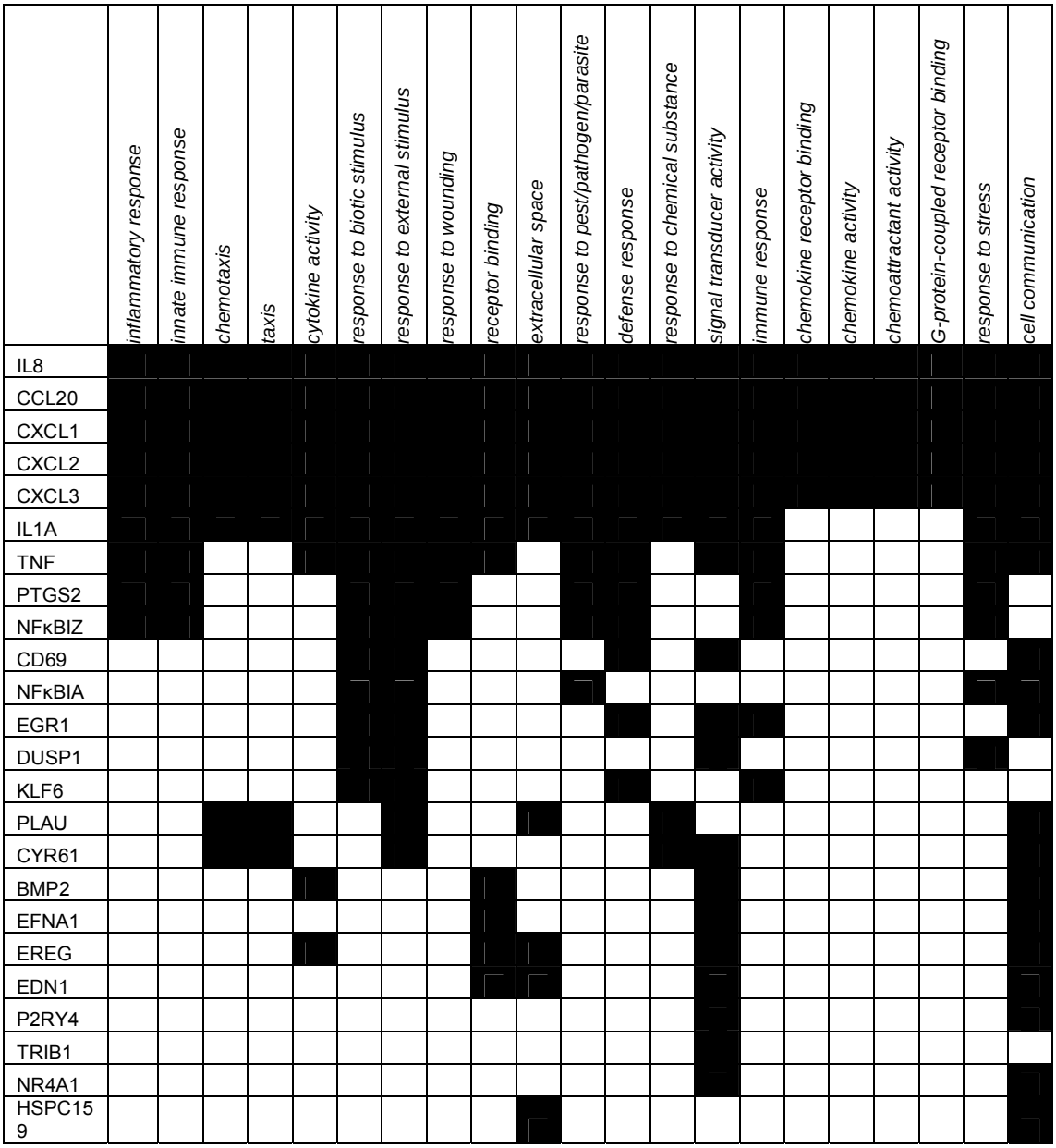


Table 5.4

Signature genes of clusters 7 and 15 which belonged to over-represented gene-ontology classifications.

5.3.5. Confirmatory Taqman PCR

In order to validate the microarray findings, Taqman qPCR of DEFB4 (the gene expressing HBD2) along with six of the signature genes from clusters 7 and 15 (IL-8, IL-1α, TNFα, BMP2, CD69 and PTGS2) was performed, in four patient samples. These genes were chosen to represent either crucial inflammatory mediators known to be expressed in pregnancy and labour (IL-8, IL-1α, TNFα, and PTGS2), or novel factors, not well described in the amnion (BMP2 and CD69). Four timepoints (1, 2, 3 and 6 hours) were examined; unfortunately RNA from the 8 hour timepoint of two samples was degraded, so this timepoint was not included in validation experiments.

To enable comparison of the patterns of gene expression in the microarray and Taqman qPCR data, two adjustments were made. Firstly, the microarray log2 ratios were transformed to express the relationship of the sample to the reference, rather than vice versa. This was done using the formula "$(1/2^{\wedge (\log2\ ref/sample)})-1$" where $2^{\wedge (\log2\ ref/sample)}$ was greater or equal to 1 and "$-2^{\wedge (\log2\ ref/sample)}$" where $2^{\wedge (\log2\ ref/sample)}$ was less than 1. Secondly, in the Taqman qPCR data, mRNA levels in IL-1β treated samples were expressed relative to the median of all the untreated controls (analogous to the pooled controls, used as the reference in the microarray).

Microarray and Taqman qPCR determined kinetics of gene expression were similar in DEFB4, IL-8, IL-1α, TNFα, BMP2 and CD69 (Figures 5.8-5.10). Expression of these genes, as determined by Taqman PCR, was also significantly greater in treated samples than controls in at least two timepoints ($p<0.05$). The Taqman qPCR determined pattern of expression of PTGS2 was slightly different from that seen in the microarray, and was not significant at any timepoint.

5.3.6 CD69 mRNA expression was increased in amnion tissue that had been exposed to labour

CD69 mRNA expression was significantly higher after normal labour (spontaneous vertex delivery) than after prelabour elective Caesarean section (Figure 5.11; $P<0.05$).

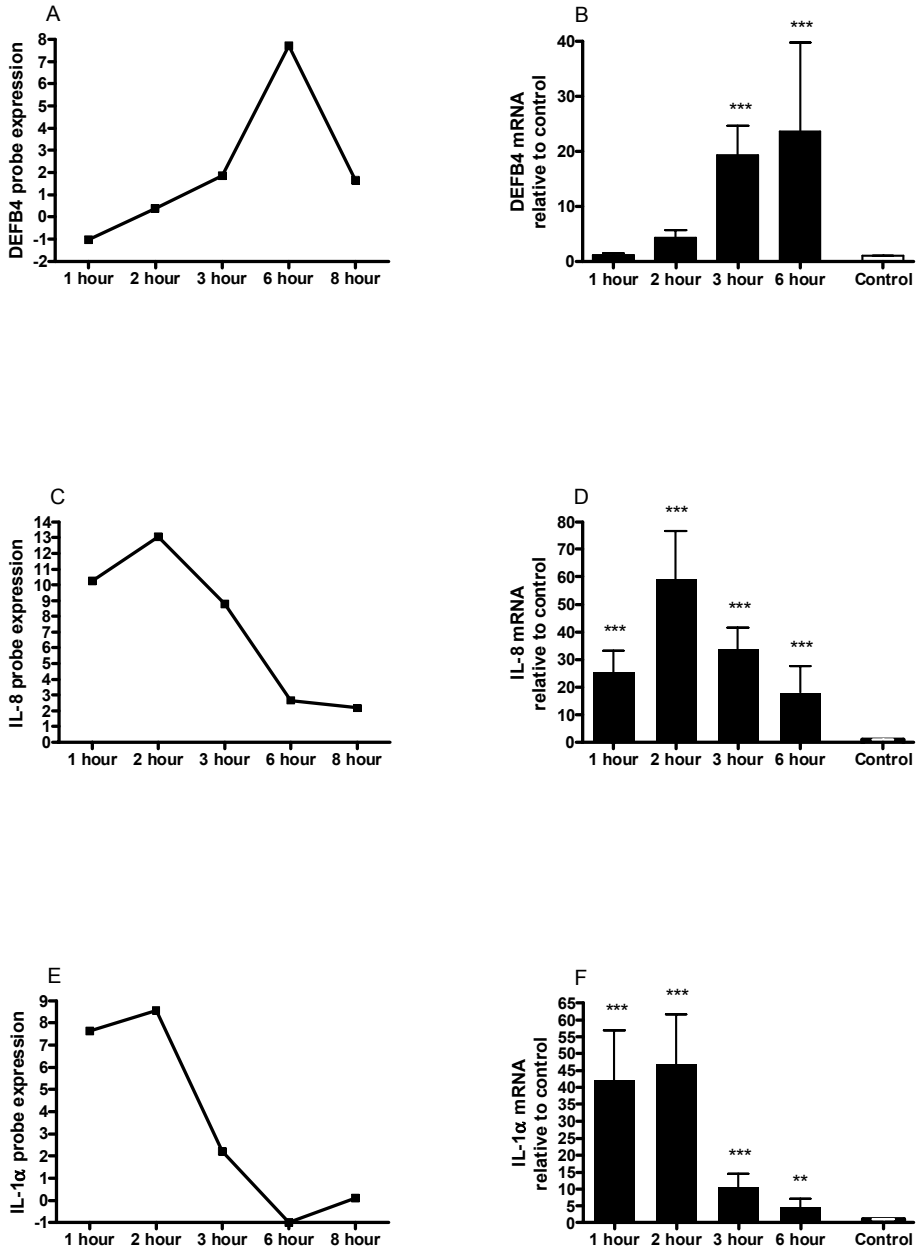


Figure 5.8

DEFB4 (HBD2) (A and B); IL-8 (C and D) and IL-1α (E and F) expression. Graphs A, C and E represent relative expression of treated sample to pooled control as determined by Agilent cDNA microarray (n=1). Graphs B,D and F represent mean +/- SEM expression of mRNA relative to the median of the timepoint controls, determined by Taqman PCR (n=4). **P<0.01 and ***P<0.001 (One-way repeated measures ANOVA with Tukey's post-test).

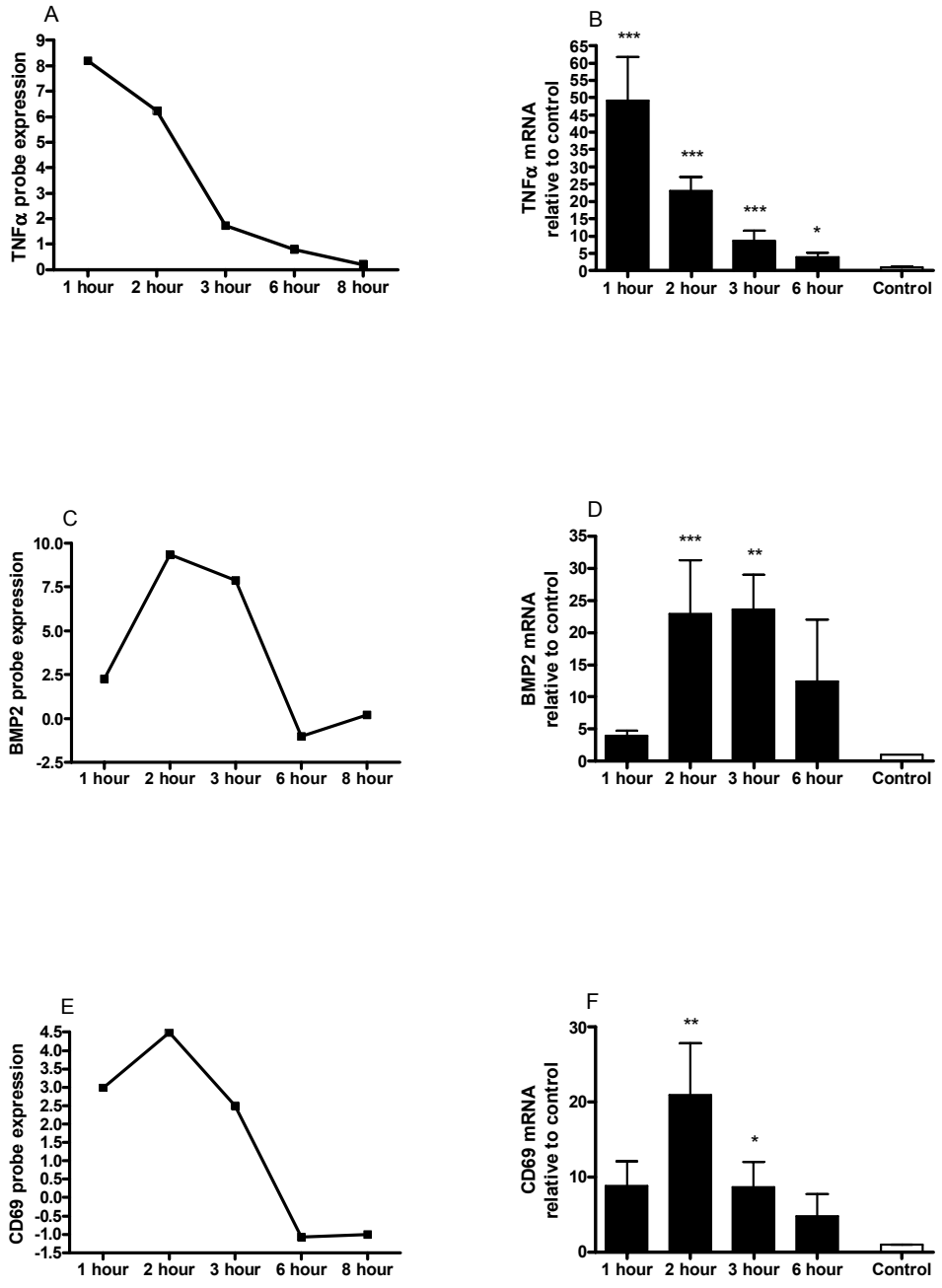


Figure 5.9

TNFα (A and B); BMP2 (C and D) and CD69 (E and F) expression. Graphs A, C and E represent relative expression of treated sample to pooled control as determined by Agilent cDNA microarray (n=1). Graphs B,D and F represent mean +/- SEM expression of mRNA relative to the median of the timepoint controls, determined by Taqman PCR (n=4). *P<0.05, **P<0.01 and ***P<0.001 (One-way repeated measures ANOVA with Tukey's post-test).

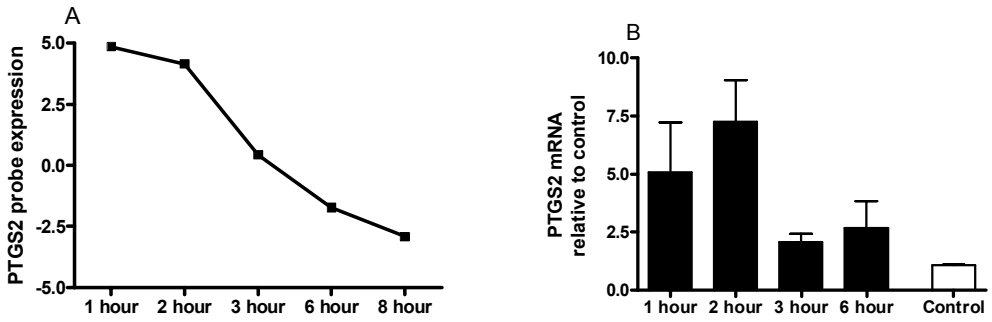


Figure 5.10

PTGS2 (COX2) expression. Graph A represents relative expression of treated sample to pooled control as determined by Agilent cDNA microarray (n=1). Graph B represents mean +/- SEM expression of mRNA relative to the median of the timepoint controls, determined by Taqman PCR (n=4).

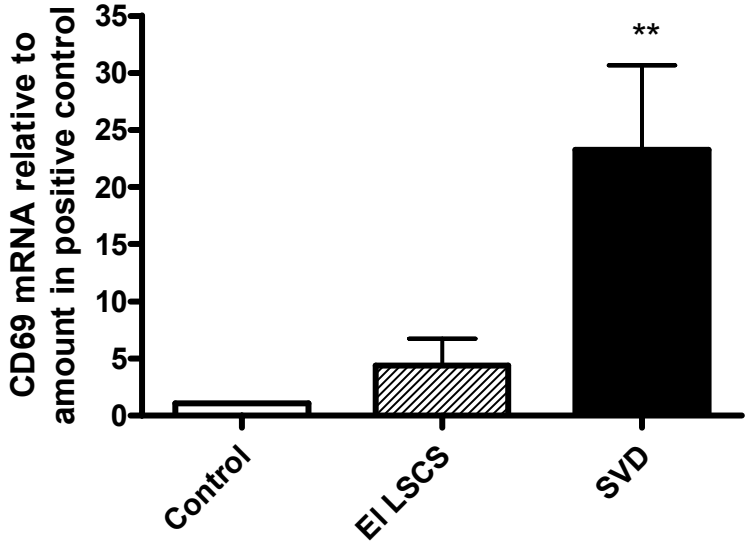


Figure 5.11

Mean +/- SEM CD69 mRNA expression in amnion tissue obtained at prelabour Caesarean section (n=6) or following normal labour (n=5), relative to positive control (primary amnion mRNA) as determined by Taqman qPCR. * $P<0.05$ (Unpaired t-test El LSCS vs SVD).

5.3.7. IL-8, BMP2, and Indomethacin do not influence HBD2 expression

The effects of IL-8 10ng/ml, BMP2 10ng/ml and endothelin-1 1nM on HBD2 expression were examined. They had no significant effect on HBD2 mRNA at 6 or 24 hours (data not shown). In addition the PTGS inhibitor indomethacin 5μM had no effect on HBD2 production, either in isolation or in conjunction with IL-1β 10ng/ml at 6 or 24 hours (data not shown).

5.3.8. EGFR inhibition has no effect on IL-1β invoked expression of HBD2

The EGF receptor inhibitor (AG1478 200nM) had no effect on IL-1β induced HBD2 expression (data not shown).

5.3.9. MAPK p38 inhibition diminishes the effect of IL-1β on HBD2

The p38 MAPK inhibitor SB203580 5μM decreased expression of HBD2 in response to IL-1β at mRNA (Figure 5.12A) at all time points. It also decreased protein expression (Figure 5.12B). In contrast, it had no demonstrable effect on IL-8 mRNA expression invoked by IL-1β (data not shown).

The ERK inhibitor (PD96059 50μM) and JNK inhibitor (JNKII inhibitor 5μM) had no effect on expression of IL-1ß induced HBD2 mRNA expression in preliminary experiments (data not shown).

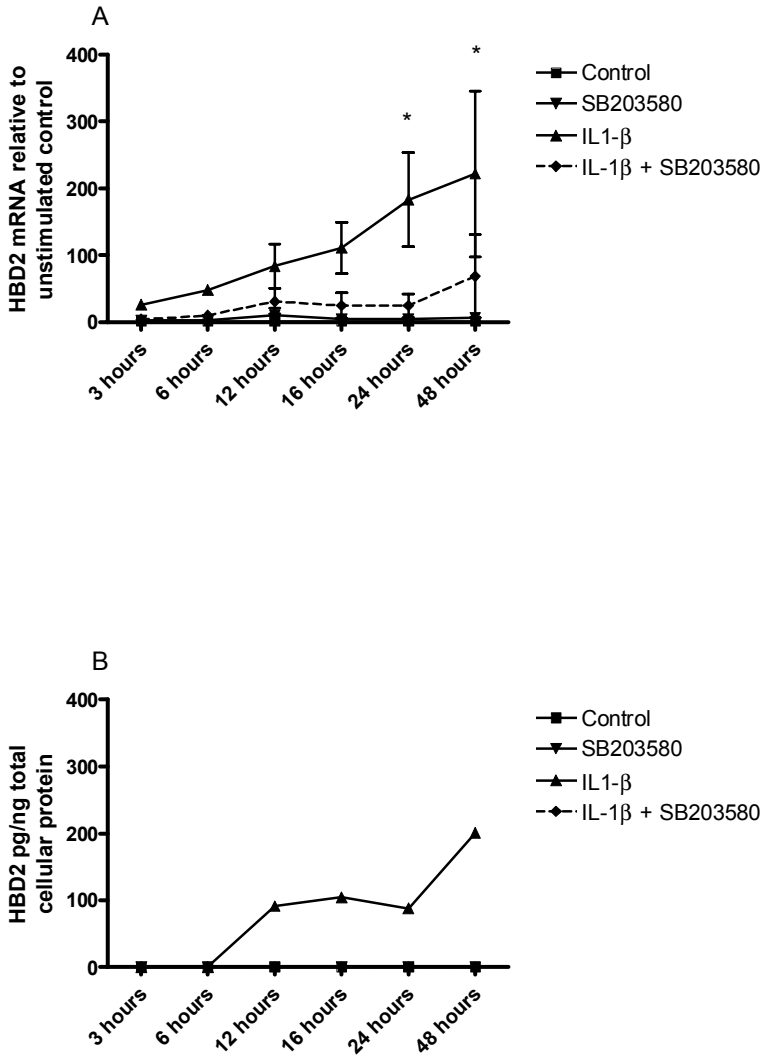


Figure 5.12

A. Mean +/- SEM expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=3), relative to amount in time matched unstimulated control, as determined by Taqman qPCR, after treatment with SB203580 5 μM and/or IL-1β 10ng/ml for 3, 6, 12, 16 or 24 hours. *P=<0.05 IL-1β vs IL-1β + SB203580 (Two-way ANOVA)

B. Mean amount of HBD2 (pg) per ng of total cellular protein secreted by primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=1), after stimulation with IL-1β 10ng/ml. Amounts were undetectable from untreated cells, or cells treated with SB2030580 5μM +/- IL-1β 10ng/ml.

5.3.10. NFκB inhibition by sulfasalazine

5.3.10.1. *Sulfasalazine diminishes the effect of IL-1β and IL-17 on HBD2*

The NFκB inhibitor sulfasalazine 5μM appeared to abrogate the effect of IL-1β on HBD2 mRNA expression in primary amnion epithelial cells, although this trend was not significant as only two samples were examined (Figure 5.13A). Sulfasalazine also diminished the effect of IL-17 on HBD2 production (Figure 5.13B).

5.3.10.2. *NFκB inhibition increases the effect of IL-1β on IL-8*

In contrast, sulfasalazine increased expression of IL-8 mRNA when compared to timematched unstimulated control (Figure 15.14). When IL-1β was applied with sulfasalazine, there appeared to be further upregulation (Figure 15.14). The addition of IL-17 to sulfasalazine did not have any effect on IL-8 expression (data not shown).

5.3.11. IL-1β and IL-17 upregulate NFκBIζ, but have differing effects on NFκBIα

5.3.11.1. *IL-1β and IL-17 increase expression of NFκBIζ*

NFκBIζ (IκB-ζ) mRNA was increased by IL-1β 10ng/ml approximately 28 fold at 2 hours and 13 fold at 3 hours, with a return towards unstimulated levels at 6 hours (Figure 5.15A). There was no further elevation over 48 hours (data not shown). IL-17 also increased NFκBIζ mRNA expression, with an approximately 7 fold rise at 2 and 3 hours (Figure 5.15B).

5.3.11.2. *IL-1β , but not IL-17, increases expression of NFκBIα*

NFκBIα was upregulated approximately 4 fold by IL-1β at 1, 2 and 3 hours, with return towards baseline values at 6 hours (Figure 5.15C). There was no further elevation over 48 hours (data not shown). In contrast, IL-17 had no effect on NFκBIα at any timepoint up to 6 hours (Figure 5.15D), or up to 48 hours (data not shown).

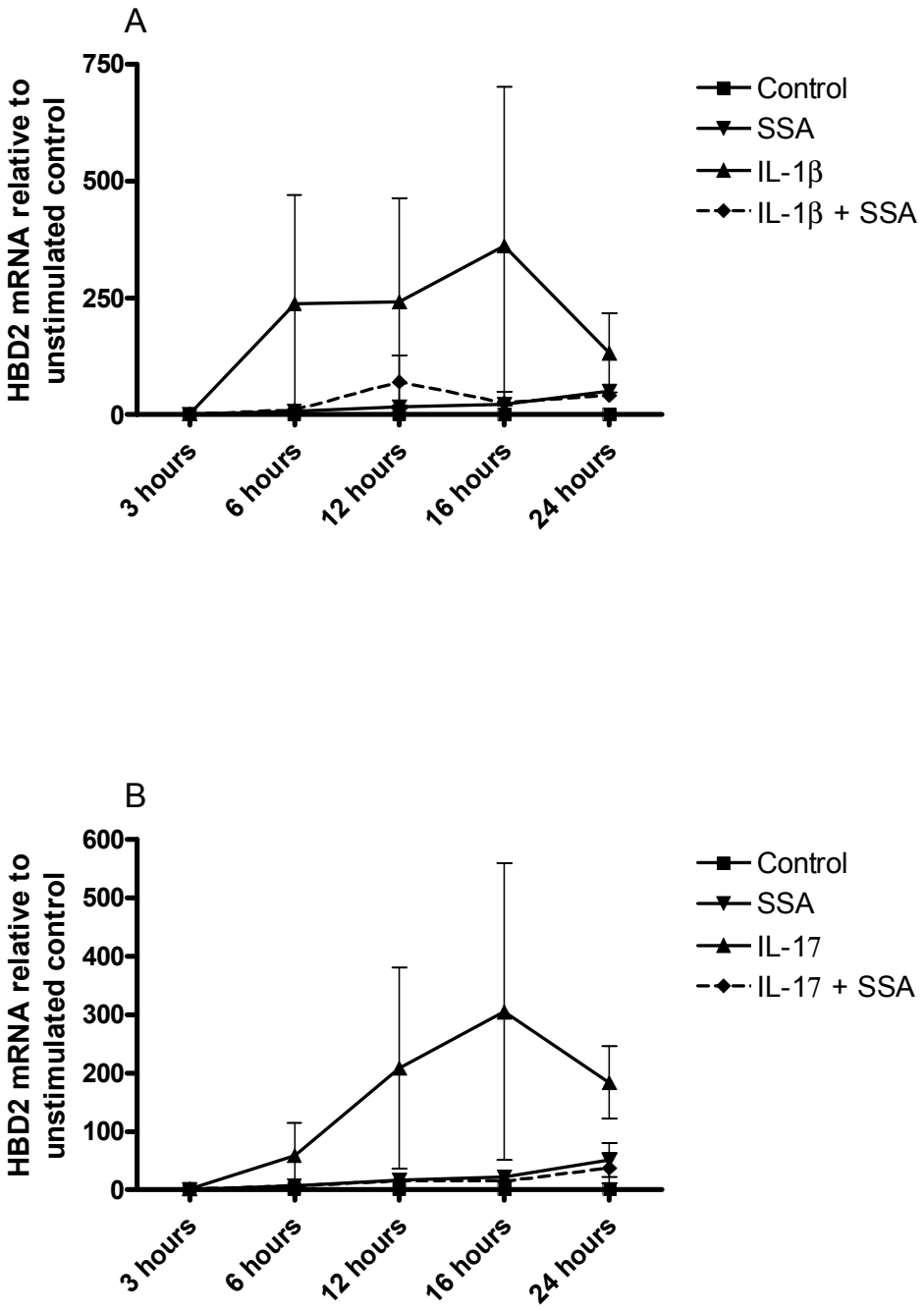


Figure 5.13

Mean +/- SEM expression of HBD2 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=2), relative to amount in time matched unstimulated control, as determined by Taqman qPCR, after treatment with sulfasalazine 5μM and/or IL-1β 10ng/ml (A) or IL-17 10ng/ml (B) for 3, 6, 12, 16 or 24 hours.

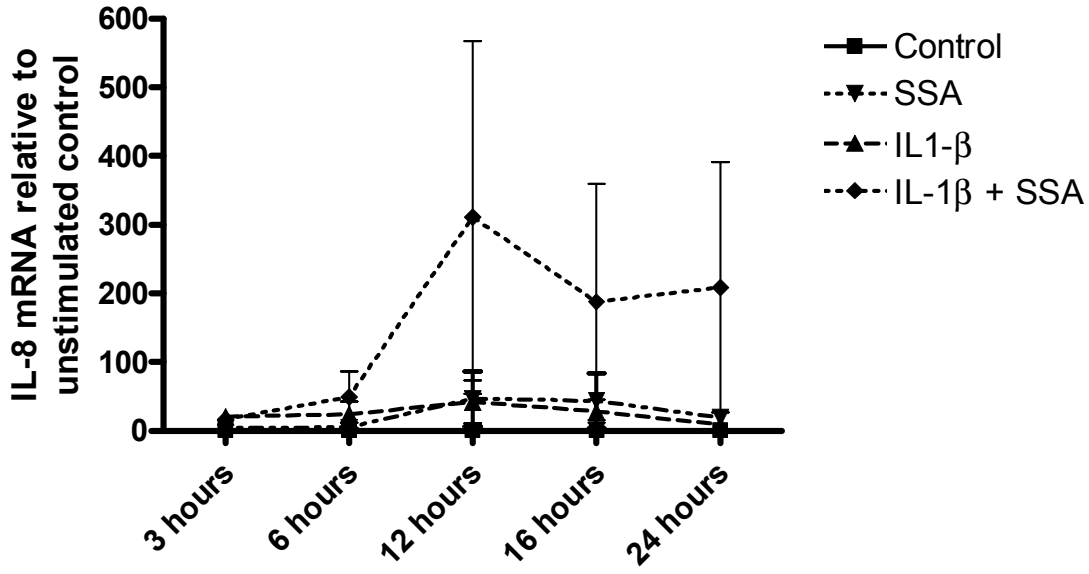


Figure 5.14

Mean +/- SEM expression of IL-8 mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=2), relative to amount in time matched unstimulated control, as determined by Taqman qPCR, after treatment with sulfasalazine 5μM and/or IL-1β 10ng/ml for 3, 6, 12, 16 or 24 hours. IL-8 expression was significantly greater than in timematched unstimulated control after treatment with sulfasalazine at 12 hours, 16 hours and 24 hours; IL-1β at 3, 6, 12, 16 and 24 hours; and IL-1β with sulfasalazine at 6, 12, 16 and 24 hours. IL-8 expression was significantly greater after treatment with IL-1β with sulfasalazine, than after sulfasalazine alone at 12 and 24 hours.

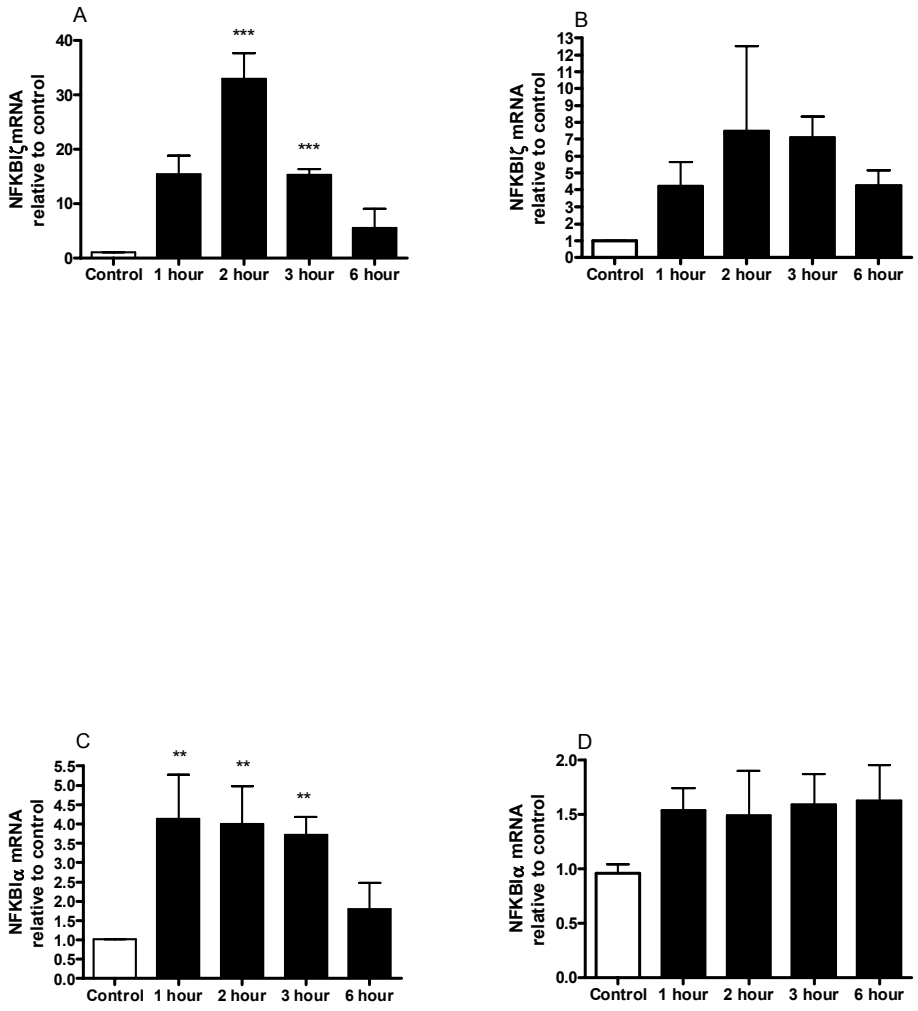


Figure 5.15

A and B. Mean +/- SEM expression of NFκBIζ mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term and treated with IL-1β 10ng/ml (A; n=5) or IL-17 10ng/ml (B; n=2) for 1, 2, 3 or 6 hours, relative to amount in time matched unstimulated control, as determined by Taqman qPCR.

C and D. Mean +/- SEM expression of NFκBIα mRNA in primary cultured amnion epithelial cells obtained at prelabour Caesarean section at term (n=2) and treated with IL-1β 10ng/ml (A; n=5) or IL-17 10ng/ml (B; n=2) for 1, 2, 3 or 6 hours, relative to amount in time matched unstimulated control, as determined by Taqman qPCR *P<0.05 **P<0.01 ***P<0.001 (One–way repeated measures ANOVA with Tukey's post-test).

5.4. DISCUSSION

The results of this chapter provide information regarding the nature of the response invoked by IL-1β in the amnion. IL-1β results in a time dependent upregulation of genes, many of which are involved in the inflammatory or immune responses. This provides pointers to molecules that may be involved in controlling the biphasic expression of HBD2. It also has more general relevance in the study of the inflammatory nature of labour, as IL-1β can stimulate labour in animal models (Romero, Mazor et al. 1991; Sadowsky, Adams et al. 2006) and is elevated in normal, and particularly infection associated preterm labour (Romero, Mazor et al. 1992; Hillier, Witkin et al. 1993; Dudley, Collmer et al. 1996; Gunn, Hardiman et al. 1996; Figueroa, Garry et al. 2005).

Experimental Design

Microarray technology was chosen as an efficient means of screening the entire genome for changes induced in amnion epithelial cells by IL-1β. Limitations of the technique must be recognised, however. The generation of false negative and false positive results can occur, especially if poor experimental design, technique or analyses are employed. Rigorous planning, standardized culture and treatment conditions, and high quality RNA were therefore enforced to maximize the significance of the results. In addition microarray standard operating procedures were employed to ensure the data was reliable and reproducible (Forster, Roy et al. 2003). All data was "Minimum Information About a Microarray Experiment" (MIAME) compliant (Brazma, Hingamp et al. 2001), meaning that standardised information about all stages of the experiment can be provided, allowing amalgamation of data from different groups and enabling advanced data mining.

Two caveats should be borne in mind when evaluating the results. Firstly, this microarray data pertains to only one biological sample. The experiment was designed to examine one patient specimen over five timepoints, rather than multiple patient samples at one time point. This approach gives maximal information from the fewest number of microarray chips. Genotypic variation in the sample responses is

excluded, and timepoints, which were relatively close together, could act as replicates corroborating genuine changes in gene expression pattern. Secondly, the six hour timepoint microarray results showed fewer upregulated probes than the other timepoints, which is a minor cause for concern, and additional caution is needed in data interpretation of these time points. These factors reinforce the need for validatory experiments.

The necessary substantiation of the microarray findings was provided by confirmatory Taqman qPCR experiments, performed on four biological replicates. Despite its limitations, Taqman qPCR is referred to as the "gold-standard" for gene expression measurements (Shi, Tong et al. 2005). Seven genes were examined in this way, which exhibited reassuringly similar expression patterns to those seen in the microarray probe sets. As expected, relative expression was higher when analyzed by Taqman qPCR than microarray. This is a recognised phenomenon, and may be secondary to the optimization of conditions for individual gene detection in Taqman qPCR, as opposed to the generalized conditions in microarrays. Additional signature genes were not examined by Taqman qPCR, as the experiments would have been costly and time-consuming, without providing much additional information for the purposes of this thesis. Added credence was given to unconfirmed findings by supporting evidence from related published studies, and physiological plausibility of the response.

The microarray timepoints were quite close to each other to ensure sequential changes in gene expression were not missed, and enable the maximum amount of valid data to be extrapolated from one biological replicate. They were chosen to provide information on factors that could, allowing for protein translation, be temporally associated with the decline in HBD2 production which occurs after an initial peak at 6 or 12 hours. This means they are limited to revealing early and intermediate changes in gene expression, but give no data on very early or late patterns, which also might be important in the HBD2 response.

The Inflammatory Response to IL-1β

The cDNA microarray suggested that the amnion has an "inflammatory footprint", producing multiple inflammatory mediators in response to IL-1β. Infusion of IL-1β has been shown to cause a sterile chorioamnionitis in non-human primates (Sadowsky, Adams et al. 2006). It seems likely that this effect would be dependent on the ability of IL-1β to elicit inflammatory cell infiltration in the fetal membranes– a theory supported by the microarray findings. IL-8, CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ) are potent neutrophil chemokines, whilst CCL20 (MIP-3α) is involved in monocyte differentiation and chemotaxis. Plasminogen activator urokinase (PLAU) and cysteine rich angiogenic-inducer 61 (CYR61) are also implicated in cellular migration, and IL-1α and TNFα are pro-inflammatory cytokines with multiple effects including cellular proliferation and differentiation.

Several of these factors have previously been identified as components of the amniotic fluid. The onset of labour at term coincides with rises in TNFα (Romero, Mazor et al. 1992) and IL-8 (Romero, Ceska et al. 1991; Laham, Rice et al. 1993; Saito, Kasahara et al. 1993). Furthermore, elevations in TNFα (Romero, Manogue et al. 1989; Laham, Brennecke et al. 1994; Stallmach, Hebisch et al. 1995; Arntzen, Kjollesdal et al. 1998; Weiyuan and Li 1998; Shobokshi and Shaarawy 2002), IL-1α (Romero, Mazor et al. 1992; Hillier, Witkin et al. 1993; Figueroa, Garry et al. 2005) IL-8 (Cherouny, Pankuch et al. 1993; Saito, Kasahara et al. 1993; Stallmach, Hebisch et al. 1995; Hsu, Meaddough et al. 1998; Shobokshi and Shaarawy 2002)and CXCL1 (Cohen, Ghezzi et al. 1996; Hsu, Meaddough et al. 1998) are evident in the amniotic fluid in spontaneous and infection-associated preterm labour. Invading leukocytes have been cited as a major source of these inflammatory mediators (Bowen, Chamley et al. 2002). The findings of this study suggest that the amnion could also be a contributor, its large surface area meaning production could be substantial. Other papers have shown increased expression of TNFα. (Dudley, Collmer et al. 1996), IL-1α (Laham, Brennecke et al. 1996) and IL-8 (Keelan, Marvin et al. 1999) in amnion tissue exposed to labour, supporting the theory of the amnion as a provider of cytokines.

Although there are no studies individually examining the amniotic production of CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ) or CCL20 (MIP-3α), they have recently been recognised in a whole genome microarray study of chorioamniotic membranes (Haddad, Tromp et al. 2006). In fact, these genes, along with IL-8 and BMP2, were found to be amongst the most discriminatory genes between samples of chorioamniotic membranes obtained from women in labour and women not in labour at term. Other discriminatory genes found, coinciding with those seen in the microarray performed in this chapter, include IL-1α, NFκBIα, TNFAIP3 and epiregulin. Similar patterns of gene expression were again identified in a medium density array of inflammatory probes which compared amnion tissue from women in spontaneous labour at term, spontaneous preterm labour, or infection associated preterm labour, with amnion from women at term and not in labour (Marvin, Keelan et al. 2002). CXCL2 (GROβ), CXCL3 (GROγ) and IL-8 were more highly expressed in amnion exposed to labour; whereas CXCL1 (GROα), CXCL2 (GROβ), IL-8 and CCL20 (MIP-3α) were upregulated in preterm labour, particularly when associated with infection. Expression of these mediators was attributed to infiltrating leukocytes (Marvin, Keelan et al. 2002). However, the exclusion of this cell type in the primary amnion culture system suggests that a direct effect of IL-1β on amnion epithelial cells may be responsible.

The microarray in this thesis did not show effects on some notable discriminatory probes found in the other microarray studies, including Pre-B-cell colony enhancing factor (PBEF), IL-6 and TLR2 (Haddad, Tromp et al. 2006) or PBEF, IL-1β and MMP14 (Marvin, Keelan et al. 2002). This suggests expression of these genes in a different cellular population to amnion epithelial cells, or that their expression is outwith the control of IL-1β. It was striking that in all three microarray studies, relatively few genes were downregulated.

PTGS2 (COX2) expression was seen to be elevated in primary amnion epithelial cells by IL-1β in the cDNA microarray. This is a well recognized response (Mitchell, Edwin et al. 1993; Trautman, Edwin et al. 1996; Allport, Pieber et al. 2001), and it is likely that the resultant production of prostaglandins is the mechanism by which IL-

1β can stimulate labour (Sadowsky, Adams et al. 2006). However, in the confirmatory PCR experiments, although there was a trend for increased expression, it was not significant. This is probably due to a disparity in the timing of the response, as a transient rise in PTGS2 was evident in every sample, but the timing of this peak varied. In addition there was quite marked variation in PTGS2 mRNA expression. This may be secondary to the subject's proximity to labour. PTGS2 in the amnion rises at the end of pregnancy and onset of labour (Slater, Dennes et al. 1999). Whilst women were included only if there were no signs of impending delivery, it is obviously impossible to know exactly how close they were to the spontaneous establishment of labour.

Other molecules

BMP2 and endothelin-1 are two other genes which were upregulated in response to IL-1β. Although these are not recognised as being part of the classical inflammatory response, they have potential of providing stimulant effects on the amnion or adjacent tissues. BMP2 has recently been discovered in the chorioamniotic membranes, where expression is increased in normal labour, and preterm labour associated with chorioamnionitis (Kim, Romero et al. 2005). This study also found BMP2 could be upregulated by IL-1β in primary amnion cells, and when it was, it co-localized with PGE_2. In addition, recombinant BMP2 was shown to stimulate PGE_2 synthesis, suggesting its involvement in parturition. Endothelin-1 has also been shown to increase prostaglandin synthesis (Benigni, Gaspari et al. 1991), and it is also a myometrial activator (Word, Kamm et al. 1990). Expression in the amnion has also been previously described (Sunnergren, Word et al. 1990), as has the ability of IL-1β to stimulate its production (Casey, Word et al. 1991; Mitchell 1991; Fried, Sand et al. 2003). It is a potent vasoconstrictor, but its role in the avascular amnion is not clearly defined. It may diffuse and have a regulatory function on the adjacent chorion, placenta or myometrium. Further clarification of the roles of these factors *in vivo* is required.

The expression of the CD69 gene in amnion cells was a new finding. It codes for a type II C-type lectin ascribed to the family of NK receptors, with an as yet,

undetermined ligand. It is expressed following activation on all bone-marrow derived cells (Testi, D'Ambrosio et al. 1994), but has never been reported in cells of epithelial origin before. Knock-out mice models have shown that CD69 deficiency leads to diminished TGF-β production in T cells, NK cells and macrophages. TGF-β is an anti-inflammatory cytokine, so this supports an enhanced immune response. The knock-out phenotype thus exhibits increased inflammation in a collagen induced arthritis model (Sancho, Gomez et al. 2003); and enhanced tumour depletion in an NK-sensitive tumour model (Esplugues, Sancho et al. 2003). However, *in vitro* experiments have shown conflicting pro-inflammatory functions of CD69, including production of TNF-α, IL-1β and RANTES, suggesting it may have a dual-role, depending on the cellular context (Sancho, Gomez et al. 2005).

In this study CD69 was repeatedly identified in primary cultured amnion epithelial cells by Taqman PCR, and expression was upregulated by IL-1β. The stromal layer of the amnion does contain a few tissue macrophages, which are mainly of fetal origin (Bulmer and Johnson 1984), however it seems unlikely that these cells would persist in cell culture at consistently high enough numbers to account for the levels of CD69 mRNA expression observed. In addition, CD69 was expressed in amnion tissue, with apparent increases seen in tissue exposed to normal labour, although this may be secondary to infiltrating leukocytes. Confirmation of expression of CD69 at protein level is an area of future investigation. Establishment of its ligand will be crucial in elucidation of a possible role in the amnion.

Factors involved in HBD2 production

It was hypothesized that a secondary gene product could be responsible for the later peak in HBD2 mRNA expression, and members of K-means clusters 7 and 15 were considered as potential candidates. Unfortunately subsequent investigations provided no evidence that these molecules were involved in the regulation of HBD2.

TNFα does increase HBD2 expression, as shown in Chapter 4 (Figure 4.8), but the magnitude of this change (5-7 fold) is much less than the peaks invoked by IL-β. As IL-1α has identical effects to IL-1β, and IL-1β was added in excess, it seems unlikely

that this mediator could further increase production of HBD2. Treatment with IL-8, BMP2 and endothelin-1 had no obvious effect on HBD2 expression. Furthermore, the PTGS inhibitor indomethacin had no effect, either on its own, or when added with IL-1β; indicating that prostaglandins, the PTGS2 products, are not an influencing factor. The EGF receptor antagonist AG1478 also did not alter the effect of IL-1β on HBD2 expression, implying EGF receptor agonists, such as epiregulin, were not responsible for the later peak in HBD2 production.

Further study focused on potential regulatory mechanisms in signalling pathways. The results of these were inconclusive on their own, but should provide directions for future investigations.

NFκB and AP-1 transcription factors have been previously implicated in IL-1β signalling in the amnion (Elliott, Allport et al. 2001; Lindstrom and Bennett 2005; Mohan, Sooranna et al. 2007) and WISH cells (Allport, Pieber et al. 2001). It was therefore reassuring that components of these pathways were represented in the microarray. NFκB and AP-1 are key regulators of the inflammatory and immune response, and activation of at least one MAPK is required, in addition to NFκB, to promote strong transcription (Kracht and Saklatvala 2002).

SB203580 is a pyridinyl imidazole that is an ATP-competitive inhibitor of p38. It was found to decrease the expression of HBD2 provoked by IL-1β. In contrast, specific inhibitors of JNK and ERK had no effect on IL-1β induced HBD2 expression. Together these results suggest that p38 MAPK, but not JNK or ERK may be involved in IL-1β induced expression of HBD2. The p38 pathway is also essential for mRNA stabilization of many inflammatory genes (Kracht and Saklatvala 2002), and this may be an additional or alternative means by which HBD2 production is regulated.

The involvement of the NFκB signalling pathway in HBD2 production was also examined using pathway inhibitors. The effect of sulfasalazine was examined, after trials with two other NFκB inhibitors (BAY 11-7082 and NFκB inhibitor, both from

Calbiochem) gave inconsistent results. This may have been due to a cytotoxic effect on the cultures, as a decrease in viable cells was seen using trypan blue exclusion. A reduction of viable cells and a decrease in mRNA quality and yield was also found with sulfasalazine treatment – but this only occurred after 48 hours of treatment. This timepoint was therefore excluded from analysis.

Sulfasalazine is a combination of the antibiotic sulfapyridine, and the anti-inflammatory 5-amino-salicycylic acid. It has been shown to be an inhibitor of NFκB activation both *in vitro* (Wahl, Liptay et al. 1998) and *in vivo* (Gan, Chen et al. 2005), and suppresses LPS-induced NFκB DNA binding activity in human gestational tissues (Lappas, Permezel et al. 2002). It is thought to inactivate IKKα (Weber, Liptay et al. 2000), thereby preventing phosphorylation of IκBα and translocation of NFκB subunits to the nucleus, although the exact mechanism of action is unconfirmed. It is recognised however, that its effects are not confined to this enzyme, and it may have other anti-inflammatory effects.

Sulfasalazine seemed to decrease expression of HBD2 in response to both IL-1β and IL-17 over 24 hours; although this was examined at mRNA level in only 2 patient samples, therefore these results must be viewed as being preliminary. Nevertheless, it is consistent with the involvement of the NFκB pathway in the production of HBD2. Further experiments should be performed to confirm this.

In contrast, sulfasalazine upregulated IL-8 mRNA expression when compared to unstimulated controls. This effect was surprising, especially as a previous study has shown that sulfasalazine downregulates the effect of IL-1β on IL-8 protein production in amnion tissue (Lappas, Permezel et al. 2002). In addition, when primary amnion cells were incubated with IL-1β with sulfasalazine, an even more dramatic elevation in IL-8 mRNA was seen. The reasons for this are unclear. The increase in expression is comparatively late, and no increase is seen at 3 or 6 hours, suggesting that sulfasalazine may interfere with the negative feedback of NFκB. One hypothesis is that it occurs via secondary inhibition of IκBα production, which itself is under the control of NFκB. An alternative theory is that sulfasalazine in some way

promotes the nuclear retention and DNA binding of NFκB. This can occur through the expression of chaperoning cofactors, such as hypophosphorylated IκBβ (Thompson, Phillips et al. 1995; Lindstrom and Bennett 2005).

The identification of NFκBIζ in primary cultured amnion epithelial cells by the microarray was a novel observation. NFκBIζ is a recently discovered cofactor of NFκB, induced by IL-1β but not TNFα (Haruta, Kato et al. 2001; Eto, Muta et al. 2003). Initial experiments found it was localized in the nucleus, and acted as a negative regulator of NFκB via prevention of DNA binding of the transcription factor (Yamazaki, Muta et al. 2001; Totzke, Essmann et al. 2006). Subsequent studies have found it is essential for expression of a subset of inflammatory genes induced by Toll-like receptor agonists and IL-1β, but not TNFα (Yamamoto, Yamazaki et al. 2004) and that it itself has transcriptional activity upon interaction with the p50 NFκB subunit, with both stimulatory and inhibitory functions (Motoyama, Yamazaki et al. 2005).

A recent study in an airway epithelial cell line has shown that expression of the natural antimicrobial neutrophil gelatinase-associated lipocalin (NGAL) is dependent on NFκBIζ, and that HBD2 may be similarly regulated (Cowland, Muta et al. 2006). This led to the hypothesis that NFκBIζ may be crucial in the regulation of HBD2 in the amnion. Attempts were made to prove this with RNA interference studies. Unfortunately, neither transfection agents nor electroporation of cells enabled significant knockdown of NFκBIζ with small interfering RNA (siRNA). RNA interference in primary cultured cells is recognized to be problematic, and further work is required to optimize a technique for use in amnion cells which may clarify the role of NFκBIζ. The role of other inhibitory molecules upregulated in the microarray, such as TNFAIP3 which is involved in terminating the NFκB response, should also be examined.

Indirect support for the involvement of NFκBIζ in HBD2 production was provided by the observation that IL-17 upregulated both NFκBIζ and HBD2 mRNA in a similar pattern to IL-1β. Interestingly, IL-17 is capable of stabilizing NFκBIζ mRNA

in a mouse fibroblast cell line, but this occurs without degradation of NFκBIα or activation of NFκB (Yamazaki, Muta et al. 2005). In the amnion, IL-17 did not induce NFκBIα mRNA- the inhibitor of NFκB, which is itself induced by NFκB forming a negative feedback loop. IL-1β on the other hand, did increase NFκBIα expression. It would thus seem that, although IL-1β and IL-17 may both activate IKK (as indicated by the abrogation of HBD2 production by sulfasalazine) and NFκBIζ, they employ different regulatory mechanisms downstream to this. This is further evidenced by the disparate response of IL-8 produced by IL-1β and IL-17 treatment (Chapter 4; Figure 4.9 D). Exactly how these processes differ is an interesting area for future study. It is well recognized that multiple levels of regulation in inflammatory signalling occur. The cellular response varies on which components are expressed, in what proportions, as well as the stimulus; and mRNA stabilization and post-transcriptional modifications are also important.

Summary

cDNA microarray is a relatively new technique which was used to investigate the response of the amnion to IL-1β. The identification of the NFκB cofactor NFκBIζ was a novel finding, and it is speculated that this may be crucial in the production of HBD2.

Important information about the inflammatory response of the amnion was gleaned. Many studies have attributed the production of inflammatory cytokines and chemokines seen in labour to invading leukocytes. These results show that the amnion may be a direct contributor, responding to IL-1β with production of a host of inflammatory mediators. In addition, the identification of CD69 is a novel finding which may add to our understanding of the immune response of this tissue.

Microarrays generate an enormous amount of information, which would be unfeasible to validate and analyze comprehensively in the context of this thesis. However, rigorous methodology and adherence to MIAME conventions ensure the maximal potential of this data in subsequent analyses.

6. General Discussion

6.1. SUMMARY OF FINDINGS

The study of natural antimicrobials is particularly relevant in reproductive biology, where infection can have devastating consequences to fertility and pregnancy. This thesis investigated natural antimicrobial production in pregnancy. Two areas were looked at: the lower genital tract, where infections can arise which can threaten the pregnancy; and the innermost fetal membrane, the amnion. The main results of this study are summarized below

- The antileukoproteinases elafin and SLPI are constituents of cervicovaginal secretions, and levels of these factors are decreased in association with the common vaginal infection bacterial vaginosis (Chapter 3).

- Cells derived from the vagina express higher amounts of elafin than cells derived from the ectocervix or endocervix (Chapter 3).

- Cells derived from the endocervix, which is normally sterile, respond to LPS challenge by increasing production of elafin and SLPI. This response is not seen in cells derived from the vagina or ectocervix; areas which host a diverse microflora (Chapter 3).

- The amnion expresses mRNA for potent natural antimicrobials HBD1, HBD2, HBD3, SLPI and elafin (Chapter 4).

- HBD2 expression is upregulated in amnion that is exposed to normal labour (Chapter 4).

- IL-1β increases HBD2 expression in amnion tissue and primary cultured amnion epithelial cells, at both mRNA and protein level (Chapter 4).

- IL-1β and IL-17 upregulate HBD2 expression in primary cultured amnion epithelial cells in a characteristic biphasic pattern (Chapter 4).

- IL-1β treatment of the amnion results in the upregulation of many inflammatory mediators, and some novel factors including the recently discovered NFκB cofactor NFκBIζ, and the T cell marker CD69 (Chapter 5).

6.2. CLINICAL APPLICATIONS AND FUTURE DIRECTIONS

In the lower genital tract in pregnancy it seems that the antileukoproteinases elafin and SLPI are the predominant natural antimicrobials, but HBD2 is also present at low levels. As discussed in Chapter 3, these are multifunctional peptides, which may have a variety of protective roles in pregnancy. In addition to being antimicrobial, they could help prevent protease mediated degradation of the fetal membranes. Furthermore, they have anti-inflammatory activities and are a point of interface with the adaptive immune system. They thus may regulate the inflammatory response in the cervix, which, if unchecked, could initiate the parturition cascade.

The clinical relevance of these factors is suggested by the fact that levels of SLPI and elafin are decreased in the cervicovaginal secretions of women with bacterial vaginosis. Bacterial vaginosis is associated with an increased risk of preterm labour, preterm rupture of membranes and miscarriage, yet results of standard antibiotic treatments have been disappointing in improving pregnancy outcome. The pleiotropic nature of elafin and SLPI mean they may be more useful therapeutic agents in the condition whilst the anatomy of the lower genital tract means that local administration of synthetic antimicrobials could be a feasible method of delivery, minimizing systemic effects.

In the lung synthetic antileukoproteinases have been effectively delivered by aerosol. They exhibit sustained half-lives in lung fluid, and have shown promising results in terms of decreasing inflammation and neutrophil elastase activity in cystic fibrosis patients (Vogelmeier, Gillissen et al. 1996). SLPI's association with elastin and elafin's adhesion domain may help their retention in tissues. Synthetic SLPI is retained *in vivo* like native peptide with less than 0.2% excreted within 5 hours of treatment (Vogelmeier, Buhl et al. 1990). Gene therapy is another possible method of delivery. Natural antimicrobial augmentation using adenovirus vectors have been

used in animal studies, however, development of this technology is at early stages (Bals, Weiner et al. 1999; Simpson, Wallace et al. 2001).

A complementary strategy could be based on modulating the efficiency of antimicrobials via acidification of vaginal secretions. SLPI and elafin have been purported to work through a "defensin-like" mechanism, dependent on ionic attraction to microbial membranes (Hiemstra, Maassen et al. 1996). Alkali milieux have been shown to inhibit the antimicrobial activity of defensins (Singh, Tack et al. 2000). The high pHs found in bacterial vaginosis may decrease the activity of antimicrobials, predisposing to bacterial overgrowth and secondary infection. A few small trials have examined use of an intravaginal acid-buffering gel in the treatment or prophylaxis of bacterial vaginosis in non-pregnant women with mixed results (Milani, Barcellona et al. 2003; Holley, Richter et al. 2004; Fiorilli, Molteni et al. 2005; Simoes, Bahamondes et al. 2006). It would be interesting to evaluate the effect of acidification on the antimicrobial capacity of cervicovaginal secretions. It could provide a simple, cheap and low risk adjuvant treatment in the management of bacterial vaginosis in pregnancy, but large scale trials would be necessary to evaluate efficacy.

An interesting finding in the *in vitro* work was the different response to LPS in vaginally derived and endocervically derived cells. The endocervix is critically situated at the interface of the non-sterile lower and sterile upper genital tract, and cervical inflammatory mediators can stimulate parturition. The cervical innate immune response is thus likely to have a crucial role in the development of preterm labour. Both epithelial and inflammatory cell compartments may be important. A recent study examined cervical leukocyte subpopulations in the second trimester, in women who had experienced a previous preterm delivery. Fewer cervical macrophages were found in women who recurrently delivered prematurely, than in those who delivered at term (Whitworth, Pafilis et al. 2007), possibly via an increase in penetration of infections. Augmentation of antimicrobial expression may be useful in such cases, and investigation of the relationship between cervical leukocytes and natural antimicrobial production is an area for further investigation.

It has been shown that the combination of a genetic polymorphism for TNFα and bacterial vaginosis is a significant risk factor for preterm birth, whereas individually neither is a risk factor (Macones, Parry et al. 2004). This confirms the importance of gene-environment interactions in the development of preterm labour. Single nucleotide polymorphisms may affect expression of natural antimicrobials. In addition, substantial individual variation in defensin gene copy number is recognized (Hollox, Armour et al. 2003). A genetically determined deficit of defensins could cause a general susceptibility to infection, which might explain the recognized association between periodontitis and preterm labour. Alternatively, it may predispose to a less favourable course of an infection; for example allowing the ascension of lower genital tract pathogens to the upper genital tract. Conversely, excessive production of defensins could overstimulate the inflammatory response via their chemotactic activities, which in itself might contribute to preterm labour. Future studies will correlate gene copy number with defensin expression in pregnancy, and assess whether variation is associated with preterm delivery. This may allow identification of a group of women at higher risk of preterm labour, to allow targeted therapy or prophylaxis.

HBD2 is upregulated in the amnion in normal labour, perhaps to increase antimicrobial protection at this vulnerable time. This illustrates the fact that natural antimicrobials may have key roles even when parturition is not actually instigated by an infection. A variety of processes can stimulate sterile inflammation and preterm labour, and synthetic antimicrobials may be useful in the management of such cases. For example, in cervical dysfunction (incompetence) the cervix cannot support the weight of the uterine contents and dilates. The cervical mucus plug, which contains exceptionally high levels of SLPI (Helmig, Uldbjerg et al. 1995) is lost. One could speculate that the resultant decrease in local elastase inhibition and antimicrobial activity may hasten fetal membrane rupture, and that topical recombinant SLPI could be beneficial. In addition, in cervical incompetence stretch of the fetal membranes can occur where the membranes prolapse through the os. As well as causing physical disruption, amniotic stretch triggers upregulation of inflammatory signalling and prostaglandin synthesis which may contribute to PPROM (Mohan, Sooranna et al.

2007). Microbial invasion of the amniotic cavity is a frequent complication of cases of PPROM, and it is likely that loss of the physical and natural antimicrobial barrier of the amnion can allow direct passage of lower genital pathogens to the uterus and fetus. The use of synthetic natural antimicrobials could be useful in this scenario, to help decrease the incidence of secondary infections. However, the pro-inflammatory and chemotactic functions of HBD2 may limit this factor's use, and further studies of its effect on the amnion, and its adjacent tissues, are crucial in its evaluation as a potential therapy.

If bacteria do penetrate lower genital tract defence mechanisms and gain access to the uterine cavity they can cause an inflammatory response in the decidua and fetal membranes. Here bacterial products and inflammatory cytokines stimulate production of prostaglandins promoting expulsion of the fetus. Preterm labour may be an evolutionary conserved response to infection, developed to optimise survival of the fetus by delivering it from a hostile intrauterine environment, and to protect the reproductive fitness of the mother (Romero, Espinoza et al. 2006). This thesis supports the idea that the amnion has a central role in this process, mediating termination of the pregnancy on exposure to overwhelming infection. The amnion directly contributes to the inflammatory response by virtue of synthesis of chemokines, cytokines and prostaglandins.

Many of the inflammatory mediators expressed by the amnion are regulated by NFκB transcription. The expression of the NFκB cofactor NFκBIζ in the amnion was a new finding, and its role in the inflammatory responses in the amnion warrants further investigation, as it may have important co-stimulatory and/or inhibitory functions. NFκB inhibition is an attractive therapeutic target in preterm labour, and it has been suggested that agents such as sulfasalazine may be a useful intervention in its management. However, one of the problems with this approach is the complex nature, multiple interactions and ubiquity of inflammatory signalling pathways. This study has shown that although sulfasalazine can downregulate some components of the inflammatory response, such as HBD2, others, such as IL-8 are paradoxically upregulated. Differing responses may be due to the myriad of adaptors and cofactors

that can modulate the response. Caution should be applied to the advocacy of NFκB inhibitors as therapy until there is fuller understanding of the controlling mechanisms, and specific inhibitors are available to minimize unwanted or deleterious side effects. Nevertheless, rapid advances in the field are being made, and future research may allow realization of this strategy.

This thesis has provided some novel findings. Expression of CD69 by the amnion was unexpected, as this is a marker of myeloid lineages, and has not been described in epithelia before. In addition the function of IL-17 in pregnancy has not been investigated, but the responsiveness of the amnion to this mediator suggests it may be an important component. Both these factors are traditionally associated with T cells and are involved in interactions between the innate and adaptive immune responses. A speculative hypothesis is that the amnion epithelium has functions akin to immune cells, enabling it to modulate both innate and adaptive immunity at the fetal-maternal interface. Subsequent investigations will study this theory and may help elucidate these findings.

6.3. CONCLUSIONS

This study has shown that epithelial natural antimicrobials are expressed by the vagina, cervix, choriodecidua and amnion. Production of SLPI and elafin in the lower genital tract may prevent ascending infections, which could threaten the establishment and maintenance of pregnancy. In the amnion HBD2 may protect the fetus at vulnerable times such as labour. Its expression is upregulated by IL-1β and IL-17 in a characteristic biphasic pattern, and it may be an important part of the inflammatory response of this tissue.

Natural antimicrobial expression is genetically determined. Individual variation could result in a propensity for infections or infectious complications which may be crucial in the development of preterm labour. Further study in this area could lead to the development of new antimicrobial therapies for the treatment of infections in

pregnancy. In addition, it may allow strategies for the recognition of women at higher risk of preterm labour, in whom targeted treatments may be beneficial.

References

Abe, T., N. Kobayashi, et al. (1991). "Expression of the secretory leukoprotease inhibitor gene in epithelial cells." J Clin Invest **87**(6): 2207-15.

Abrahams, V. M., P. Bole-Aldo, et al. (2004). "Divergent trophoblast responses to bacterial products mediated by TLRs." J Immunol **173**(7): 4286-96.

Ackerman, W. E. T., L. H. Hughes, et al. (2005). "In Situ Immunolabeling Allows for Detailed Localization of Prostaglandin Synthesizing Enzymes Within Amnion Epithelium." Placenta.

Ackerman, W. E. T., B. H. Rovin, et al. (2004). "Epidermal growth factor and interleukin-1beta utilize divergent signaling pathways to synergistically upregulate cyclooxygenase-2 gene expression in human amnion-derived WISH cells." Biol Reprod **71**(6): 2079-86.

Ackerman, W. E. T., X. L. Zhang, et al. (2005). "Modulation of cytokine-induced cyclooxygenase 2 expression by PPARG ligands through NFkappaB signal disruption in human WISH and amnion cells." Biol Reprod **73**(3): 527-35.

Adams, K. M., J. Lucas, et al. (2006). "LPS Induces Translocation of TLR4 in Amniotic Epithelium." Placenta.

Aderem, A. and R. J. Ulevitch (2000). "Toll-like receptors in the induction of the innate immune response." Nature **406**(6797): 782-7.

Allport, V. C., D. Pieber, et al. (2001). "Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'." Mol Hum Reprod **7**(6): 581-6.

Allport, V. C., D. M. Slater, et al. (2000). "NF-kappaB and AP-1 are required for cyclo-oxygenase 2 gene expression in amnion epithelial cell line (WISH)." Mol Hum Reprod **6**(6): 561-5.

Alvi, S. A., N. L. Brown, et al. (1999). "Corticotrophin-releasing hormone and platelet-activating factor induce transcription of the type-2 cyclo-oxygenase gene in human fetal membranes." Mol Hum Reprod **5**(5): 476-80.

Ammala, M., T. Nyman, et al. (1997). "The interleukin-1 system in gestational tissues at term: effect of labour." Placenta **18**(8): 717-23.

Amsel, R., P. A. Totten, et al. (1983). "Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations." Am J Med **74**(1): 14-22.

Anderson, W. H., T. M. Davidson, et al. (1996). "Mast cell TNF mRNA expression in nasal mucosa demonstrated by in situ hybridization: a comparison of mast cell detection methods." J Immunol Methods **189**(2): 145-55.

Angelov, N., N. Moutsopoulos, et al. (2004). "Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor." Thromb Haemost **92**(2): 288-97.

Arias, F., Tomich, P. (1982). "Etiology and outcome of lowbirth weight and preterm infants." Obstet Gynecol **60**(3): 277-81.

Arntzen, K. J., A. M. Kjollesdal, et al. (1998). "TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor." J Perinat Med **26**(1): 17-26.

Ashcroft, G. S., K. Lei, et al. (2000). "Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing." Nat Med **6**(10): 1147-53.

Baergen, R., K. Benirschke, et al. (1994). "Cytokine expression in the placenta. The role of interleukin 1 and interleukin 1 receptor antagonist expression in chorioamnionitis and parturition." Arch Pathol Lab Med **118**(1): 52-5.

Baggiolini, M. (2001). "Chemokines in pathology and medicine." J Intern Med **250**(2): 91-104.

Bailey, J. V., C. Farquhar, et al. (2004). "Bacterial vaginosis in lesbians and bisexual women." Sex Transm Dis **31**(11): 691-4.

Baker, A. H., D. R. Edwards, et al. (2002). "Metalloproteinase inhibitors: biological actions and therapeutic opportunities." J Cell Sci **115**(Pt 19): 3719-27.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol **14**: 649-83.

Bals, R. (2000). "Epithelial antimicrobial peptides in host defense against infection." Respir Res **1**(3): 141-50.

Bals, R., D. J. Weiner, et al. (1999). "Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide." Infect Immun **67**(11): 6084-9.

Balu, R. B., D. A. Savitz, et al. (2002). "Bacterial vaginosis and vaginal fluid defensins during pregnancy." Am J Obstet Gynecol **187**(5): 1267-71.

Balu, R. B., D. A. Savitz, et al. (2003). "Bacterial vaginosis, vaginal fluid neutrophil defensins, and preterm birth." Obstet Gynecol **101**(5 Pt 1): 862-8.

Basso, B., F. Gimenez, et al. (2005). "IL-1beta, IL-6 and IL-8 levels in gyneco-obstetric infections." Infect Dis Obstet Gynecol **13**(4): 207-11.

Bearfield, C., E. S. Davenport, et al. (2002). "Possible association between amniotic fluid micro-organism infection and microflora in the mouth." Bjog **109**(5): 527-33.

Benigni, A., F. Gaspari, et al. (1991). "Human placenta expresses endothelin gene and corresponding protein is excreted in urine in increasing amounts during normal pregnancy." Am J Obstet Gynecol **164**(3): 844-8.

Bennett, P. R., M. P. Rose, et al. (1987). "Preterm labor: stimulation of arachidonic acid metabolism in human amnion cells by bacterial products." Am J Obstet Gynecol **156**(3): 649-55.

Bennett, W. A., S. Lagoo-Deenadayalan, et al. (1996). "Cytokine expression by models of human trophoblast as assessed by a semiquantitative reverse transcription-polymerase chain reaction technique." Am J Reprod Immunol **36**(5): 285-94.

Bennett, W. A., S. Lagoo-Deenadayalan, et al. (1998). "Cytokine expression by first-trimester human chorionic villi." Am J Reprod Immunol **40**(5): 309-18.

Bennett, W. A., S. Lagoo-Deenadayalan, et al. (1999). "First-trimester human chorionic villi express both immunoregulatory and inflammatory cytokines: a role for interleukin-10 in regulating the cytokine network of pregnancy." Am J Reprod Immunol **41**(1): 70-8.

Bennett, W. A., D. A. Terrone, et al. (2000). "Intrauterine endotoxin infusion in rat pregnancy induces preterm delivery and increases placental prostaglandin F2alpha metabolite levels." Am J Obstet Gynecol **182**(6): 1496-501.

Bensch, K. W., M. Raida, et al. (1995). "hBD-1: a novel beta-defensin from human plasma." FEBS Lett **368**(2): 331-5.
Benyo, D. F., T. M. Miles, et al. (1997). "Hypoxia stimulates cytokine production by villous explants from the human placenta." J Clin Endocrinol Metab **82**(5): 1582-8.
Berkowitz, R. S., H. M. Faris, et al. (1990). "Localization of leukocytes and cytokines in chorionic villi of normal placentas and complete hydatidiform moles." Gynecol Oncol **37**(3): 396-400.
Biggio, J. R., Jr., P. S. Ramsey, et al. (2005). "Midtrimester amniotic fluid matrix metalloproteinase-8 (MMP-8) levels above the 90th percentile are a marker for subsequent preterm premature rupture of membranes." Am J Obstet Gynecol **192**(1): 109-13.
Bingle, L., T. D. Tetley, et al. (2001). "Cytokine-mediated induction of the human elafin gene in pulmonary epithelial cells is regulated by nuclear factor-kappaB." Am J Respir Cell Mol Biol **25**(1): 84-91.
Biragyn, A., P. A. Ruffini, et al. (2002). "Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2." Science **298**(5595): 1025-9.
Bohm, B., T. Aigner, et al. (1992). "The serine-protease inhibitor of cartilage matrix is not a chondrocytic gene product." Eur J Biochem **207**(2): 773-9.
Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends Immunol **25**(6): 280-8.
Boudier, C. and J. G. Bieth (1992). "The proteinase: mucus proteinase inhibitor binding stoichiometry." J Biol Chem **267**(7): 4370-5.
Bourne, G. (1962). "The foetal membranes. A review of the anatomy of normal amnion and chorion and some aspects of their function." Postgrad Med J **38**: 193-201.
Bowdish, D. M., D. J. Davidson, et al. (2006). "Immunomodulatory properties of defensins and cathelicidins." Curr Top Microbiol Immunol **306**: 27-66.
Bowen, J. M., L. Chamley, et al. (2002). "Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition." Placenta **23**(4): 257-73.
Bowen, J. M., L. Chamley, et al. (2002). "Cytokines of the placenta and extra-placental membranes: biosynthesis, secretion and roles in establishment of pregnancy in women." Placenta **23**(4): 239-56.
Brazma, A., P. Hingamp, et al. (2001). "Minimum information about a microarray experiment (MIAME)-toward standards for microarray data." Nat Genet **29**(4): 365-71.
Brown, A. G., R. S. Leite, et al. (2004). "Mechanisms underlying 'functional' progesterone withdrawal at parturition." Ann N Y Acad Sci **1034**: 36-49.
Bryant-Greenwood, G. D. (1998). "The extracellular matrix of the human fetal membranes: structure and function." Placenta **19**(1): 1-11.
Bryant-Greenwood, G. D. and L. K. Millar (2000). "Human fetal membranes: their preterm premature rupture." Biol Reprod **63**(6): 1575-9.
Bryant-Greenwood, G. D. and S. Y. Yamamoto (1995). "Control of peripartal collagenolysis in the human chorion-decidua." Am J Obstet Gynecol **172**(1 Pt 1): 63-70.

Buhimschi, I. A., C. S. Buhimschi, et al. (2003). "Protective effect of N-acetylcysteine against fetal death and preterm labor induced by maternal inflammation." Am J Obstet Gynecol **188**(1): 203-8.

Buhimschi, I. A., M. Jabr, et al. (2004). "The novel antimicrobial peptide beta3-defensin is produced by the amnion: A possible role of the fetal membranes in innate immunity of the amniotic cavity." Am J Obstet Gynecol **191**(5): 1678-1687.

Buhimschi, I. A., M. Jabr, et al. (2004). "The novel antimicrobial peptide beta3-defensin is produced by the amnion: a possible role of the fetal membranes in innate immunity of the amniotic cavity." Am J Obstet Gynecol **191**(5): 1678-87.

Bulmer, J. N. and P. M. Johnson (1984). "Macrophage populations in the human placenta and amniochorion." Clin Exp Immunol **57**(2): 393-403.

Butler, M. W., I. Robertson, et al. (2006). "Elafin prevents lipopolysaccharide-induced AP-1 and NF-kappaB activation via an effect on the ubiquitin-proteasome pathway." J Biol Chem **281**(46): 34730-5.

Caivano, M., B. Gorgoni, et al. (2001). "The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors." J Biol Chem **276**(52): 48693-701.

Cario, E., D. Brown, et al. (2002). "Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium." Am J Pathol **160**(1): 165-73.

Cario, E. and D. K. Podolsky (2005). "Intestinal epithelial TOLLerance versus inTOLLerance of commensals." Mol Immunol **42**(8): 887-93.

Casey, M. L. and P. C. MacDonald (1996). "Interstitial collagen synthesis and processing in human amnion: a property of the mesenchymal cells." Biol Reprod **55**(6): 1253-60.

Casey, M. L., R. A. Word, et al. (1991). "Endothelin-1 gene expression and regulation of endothelin mRNA and protein biosynthesis in avascular human amnion. Potential source of amniotic fluid endothelin." J Biol Chem **266**(9): 5762-8.

Cassell, G. H., R. O. Davis, et al. (1983). "Isolation of Mycoplasma hominis and Ureaplasma urealyticum from amniotic fluid at 16-20 weeks of gestation: potential effect on outcome of pregnancy." Sex Transm Dis **10**(4 Suppl): 294-302.

Cauci, S. (2004). "Vaginal Immunity in Bacterial Vaginosis." Curr Infect Dis Rep **6**(6): 450-456.

Cauci, S., S. Driussi, et al. (1998). "Immunoglobulin A response against Gardnerella vaginalis hemolysin and sialidase activity in bacterial vaginosis." Am J Obstet Gynecol **178**(3): 511-5.

Cauci, S., S. Guaschino, et al. (2003). "Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women." Mol Hum Reprod **9**(1): 53-8.

Celik, H. and A. Ayar (2002). "Effects of erythromycin on pregnancy duration and birth weight in lipopolysaccharide-induced preterm labor in pregnant rats." Eur J Obstet Gynecol Reprod Biol **103**(1): 22-5.

Chadebech, P., D. Goidin, et al. (2003). "Use of human reconstructed epidermis to analyze the regulation of beta-defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS." Cell Biol Toxicol **19**(5): 313-24.
Challis, J. R., S. J. Lye, et al. (2001). "Understanding preterm labor." Ann N Y Acad Sci **943**: 225-34.
Challis, J. R., D. M. Sloboda, et al. (2002). "Prostaglandins and mechanisms of preterm birth." Reproduction **124**(1): 1-17.
Challis, J. R. G., S. G. Matthews, et al. (2000). "Endocrine and paracrine regulation of birth at term and preterm." Endocr Rev **21**(5): 514-50.
Chaouat, G., S. Zourbas, et al. (2002). "A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy." J Reprod Immunol **53**(1-2): 241-56.
Chapman, N. R., G. N. Europe-Finner, et al. (2004). "Expression and deoxyribonucleic acid-binding activity of the nuclear factor kappaB family in the human myometrium during pregnancy and labor." J Clin Endocrinol Metab **89**(11): 5683-93.
Chen, H. L., Y. P. Yang, et al. (1991). "Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation." Am J Pathol **139**(2): 327-35.
Chen, X., F. Niyonsaba, et al. (2007). "Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability." Eur J Immunol **37**(2): 434-44.
Chen, X., F. Niyonsaba, et al. (2005). "Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli." J Dermatol Sci **40**(2): 123-32.
Cherouny, P. H., G. A. Pankuch, et al. (1993). "Neutrophil attractant/activating peptide-1/interleukin-8: association with histologic chorioamnionitis, preterm delivery, and bioactive amniotic fluid leukoattractants." Am J Obstet Gynecol **169**(5): 1299-303.
Chow, L. and S. J. Lye (1994). "Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor." Am J Obstet Gynecol **170**(3): 788-95.
Chwalisz, K., M. Benson, et al. (1994). "Cervical ripening with the cytokines interleukin 8, interleukin 1 beta and tumour necrosis factor alpha in guinea-pigs." Hum Reprod **9**(11): 2173-81.
Cohen, J., F. Ghezzi, et al. (1996). "GRO alpha in the fetomaternal and amniotic fluid compartments during pregnancy and parturition." Am J Reprod Immunol **35**(1): 23-9.
Condon, J. C., D. B. Hardy, et al. (2006). "Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function." Mol Endocrinol **20**(4): 764-75.
Condon, J. C., P. Jeyasuria, et al. (2003). "A decline in the levels of progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of parturition." Proc Natl Acad Sci U S A **100**(16): 9518-23.

Cowland, J. B., T. Muta, et al. (2006). "IL-1beta-Specific Up-Regulation of Neutrophil Gelatinase-Associated Lipocalin Is Controlled by I{kappa}B-{zeta}." J Immunol **176**(9): 5559-66.

Crankshaw, D. J. and R. Dyal (1994). "Effects of some naturally occurring prostanoids and some cyclooxygenase inhibitors on the contractility of the human lower uterine segment in vitro." Can J Physiol Pharmacol **72**(8): 870-4.

Culhane, J. F., P. Nyirjesy, et al. (2006). "Variation in vaginal immune parameters and microbial hydrolytic enzymes in bacterial vaginosis positive pregnant women with and without Mobiluncus species." Am J Obstet Gynecol **195**(2): 516-21

Davies, J. K., R. H. Shikes, et al. (2000). "Histologic inflammation in the maternal and fetal compartments in a rabbit model of acute intra-amniotic infection." Am J Obstet Gynecol **183**(5): 1088-93.

Dempsey, P. W., S. A. Vaidya, et al. (2003). "The art of war: Innate and adaptive immune responses." Cell Mol Life Sci **60**(12): 2604-21.

Denison, F. C., A. A. Calder, et al. (1999). "The action of prostaglandin E2 on the human cervix: stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor." Am J Obstet Gynecol **180**(3 Pt 1): 614-20.

Denison, F. C., R. W. Kelly, et al. (1998). "Cytokine secretion by human fetal membranes, decidua and placenta at term." Hum Reprod **13**(12): 3560-5.

Denison, F. C., R. W. Kelly, et al. (1999). "Secretory leukocyte protease inhibitor concentration increases in amniotic fluid with the onset of labour in women: characterization of sites of release within the uterus." J Endocrinol **161**(2): 299-306.

Deniz, G., S. E. Christmas, et al. (1996). "Soluble mediators and cytokines produced by human CD3- leucocyte clones from decidualized endometrium." Immunology **87**(1): 92-8.

Dennis, G., Jr., B. T. Sherman, et al. (2003). "DAVID: Database for Annotation, Visualization, and Integrated Discovery." Genome Biol **4**(5): P3.

Diaz-Cueto, L., A. Cuica-Flores, et al. (2005). "Genetic variation in the interleukin-8 gene promoter and vaginal concentrations of interleukin-8 are not associated with bacterial vaginosis during pregnancy." J Reprod Immunol **66**(2): 151-60.

Dietze, S. C., C. P. Sommerhoff, et al. (1990). "Inhibition of histamine release from human mast cells ex vivo by natural and synthetic chymase inhibitors." Biol Chem Hoppe Seyler **371 Suppl**: 75-9.

Dinarello, C. A. (1996). "Biologic basis for interleukin-1 in disease." Blood **87**(6): 2095-147.

Ding, A., N. Thieblemont, et al. (1999). "Secretory leukocyte protease inhibitor interferes with uptake of lipopolysaccharide by macrophages." Infect Immun **67**(9): 4485-9.

Doh, K., P. T. Barton, et al. (2004). "Differential vaginal expression of interleukin-1 system cytokines in the presence of Mycoplasma hominis and Ureaplasma urealyticum in pregnant women." Infect Dis Obstet Gynecol **12**(2): 79-85.

Donders, G. G., E. Bosmans, et al. (2000). "Pathogenesis of abnormal vaginal bacterial flora." Am J Obstet Gynecol **182**(4): 872-8.

Draper, D. L., D. V. Landers, et al. (2000). "Levels of vaginal secretory leukocyte protease inhibitor are decreased in women with lower reproductive tract infections." Am J Obstet Gynecol **183**(5): 1243-8.

Dudley, D. J., D. Collmer, et al. (1996). "Inflammatory cytokine mRNA in human gestational tissues: implications for term and preterm labor." J Soc Gynecol Investig **3**(6): 328-35.

Dutheil, N., O. Malhomme, et al. (1997). "Presence of integrated DNA sequences of adeno-associated virus type 2 in four cell lines of human embryonic origin." J Gen Virol **78 (Pt 11)**: 3039-43.

Elliott, C. L., V. C. Allport, et al. (2001). "Nuclear factor-kappa B is essential for up-regulation of interleukin-8 expression in human amnion and cervical epithelial cells." Mol Hum Reprod **7**(8): 787-90.

Elliott, C. L., R. W. Kelly, et al. (1998). "Regulation of interleukin 8 production in the term human placenta during labor and by antigestagens." Am J Obstet Gynecol **179**(1): 215-20.

Elovitz, M. A. and C. Mrinalini (2004). "Animal models of preterm birth." Trends Endocrinol Metab **15**(10): 479-87.

Elovitz, M. A., Z. Wang, et al. (2003). "A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4." Am J Pathol **163**(5): 2103-11.

Espinoza, J., R. Romero, et al. (2002). "Lipopolysaccharide-binding protein in microbial invasion of the amniotic cavity and human parturition." J Matern Fetal Neonatal Med **12**(5): 313-21.

Esplugues, E., D. Sancho, et al. (2003). "Enhanced antitumor immunity in mice deficient in CD69." J Exp Med **197**(9): 1093-106.

Eto, A., T. Muta, et al. (2003). "Essential roles for NF-kappa B and a Toll/IL-1 receptor domain-specific signal(s) in the induction of I kappa B-zeta." Biochem Biophys Res Commun **301**(2): 495-501.

Farquhar, C., T. C. VanCott, et al. (2002). "Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk." J Infect Dis **186**(8): 1173-6.

Fata, J. E., A. T. Ho, et al. (2000). "Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors." Cell Mol Life Sci **57**(1): 77-95.

Fazeli, A., C. Bruce, et al. (2005). "Characterization of Toll-like receptors in the female reproductive tract in humans." Hum Reprod **20**(5): 1372-8.

Fellermann, K., D. E. Stange, et al. (2006). "A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn's disease of the colon." Am J Hum Genet **79**(3): 439-48.

Feng, Y., X. Pan, et al. (2003). "[The human beta-defensins expression in female genital tract and pregnancy-related tissues]." Sichuan Da Xue Xue Bao Yi Xue Ban **34**(2): 217-9.

Fernie-King, B. A., D. J. Seilly, et al. (2002). "Streptococcal inhibitor of complement inhibits two additional components of the mucosal innate immune system: secretory leukocyte proteinase inhibitor and lysozyme." Infect Immun **70**(9): 4908-16.

Fichorova, R. N. and D. J. Anderson (1999). "Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells." Biol Reprod **60**(2): 508-14.
Fichorova, R. N., J. G. Rheinwald, et al. (1997). "Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins." Biol Reprod **57**(4): 847-55.
Figueroa, R., D. Garry, et al. (2005). "Evaluation of amniotic fluid cytokines in preterm labor and intact membranes." J Matern Fetal Neonatal Med **18**(4): 241-7.
Fiorilli, A., B. Molteni, et al. (2005). "Successful treatment of bacterial vaginosis with a policarbophil-carbopol acidic vaginal gel: results from a randomised double-blind, placebo-controlled trial." Eur J Obstet Gynecol Reprod Biol **120**(2): 202-5.
Fitch, P. M., A. Roghanian, et al. (2006). "Human neutrophil elastase inhibitors in innate and adaptive immunity." Biochem Soc Trans **34**(Pt 2): 279-82.
Fleming, D. C., A. E. King, et al. (2003). "Hormonal contraception can suppress natural antimicrobial gene transcription in human endometrium." Fertil Steril **79**(4): 856-63.
Flynn, A., J. H. Finke, et al. (1982). "Placental mononuclear phagocytes as a source of interleukin-1." Science **218**(4571): 475-7.
Flynn, A., J. H. Finke, et al. (1985). "Comparison of interleukin 1 production by adherent cells and tissue pieces from human placenta." Immunopharmacology **9**(1): 19-26.
Forster, T., D. Roy, et al. (2003). "Experiments using microarray technology: limitations and standard operating procedures." J Endocrinol **178**(2): 195-204.
Fortunato, S. J. and R. Menon (2003). "IL-1 beta is a better inducer of apoptosis in human fetal membranes than IL-6." Placenta **24**(10): 922-8.
Fortunato, S. J., R. Menon, et al. (1994). "Expression of TNF-alpha and TNFR p55 in cultured amniochorion." Am J Reprod Immunol **32**(3): 188-93.
Fortunato, S. J., R. Menon, et al. (1995). "Amniochorion: a source of interleukin-8." Am J Reprod Immunol **34**(3): 156-62.
Franken, C., C. J. Meijer, et al. (1989). "Tissue distribution of antileukoprotease and lysozyme in humans." J Histochem Cytochem **37**(4): 493-8.
Fried, G., A. Sand, et al. (2003). "Endothelin-1 and macrophage colony-stimulating factor are co-localized in human amnion membrane cells and secreted into amniotic fluid." Mol Hum Reprod **9**(11): 719-24.
Gan, H. T., Y. Q. Chen, et al. (2005). "Sulfasalazine inhibits activation of nuclear factor-kappaB in patients with ulcerative colitis." J Gastroenterol Hepatol **20**(7): 1016-24.
Ganz, T. (2003). "Defensins: antimicrobial peptides of innate immunity." Nat Rev Immunol **3**(9): 710-20.
Garcia, J. R., F. Jaumann, et al., Eds. (2001). Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell Tissue Res. **15**(10): 1819-21

Garcia, J. R., A. Krause, et al. (2001). "Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity." Faseb J **15**(10): 1819-21.
Gardella, C., J. Hitti, et al. (2001). "Amniotic fluid lipopolysaccharide-binding protein and soluble CD14 as mediators of the inflammatory response in preterm labor." Am J Obstet Gynecol **184**(6): 1241-8.
Gardella, C., D. E. Riley, et al. (2004). "Identification and sequencing of bacterial rDNAs in culture-negative amniotic fluid from women in premature labor." Am J Perinatol **21**(6): 319-23.
Genc, M. R., S. Gerber, et al. (2002). "Polymorphism in the interleukin-1 gene complex and spontaneous preterm delivery." Am J Obstet Gynecol **187**(1): 157-63.
Genc, M. R., S. Vardhana, et al. (2004). "Relationship between a toll-like receptor-4 gene polymorphism, bacterial vaginosis-related flora and vaginal cytokine responses in pregnant women." Eur J Obstet Gynecol Reprod Biol **116**(2): 152-6.
Ghidini, A., C. B. Jenkins, et al. (1997). "Elevated amniotic fluid interleukin-6 levels during the early second trimester are associated with greater risk of subsequent preterm delivery." Am J Reprod Immunol **37**(3): 227-31.
Gibb, W. (1998). "The role of prostaglandins in human parturition." Ann Med **30**(3): 235-41.
Gibbs, R. S., R. S. McDuffie, Jr., et al. (2004). "Experimental intrauterine infection with Prevotella bivia in New Zealand White rabbits." Am J Obstet Gynecol **190**(4): 1082-6.
Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-4.
Goepfert, A. R., M. Varner, et al. (2005). "Differences in inflammatory cytokine and Toll-like receptor genes and bacterial vaginosis in pregnancy." Am J Obstet Gynecol **193**(4): 1478-85.
Goldenberg, R. L., M. A. Klebanoff, et al. (1996). "Bacterial colonization of the vagina during pregnancy in four ethnic groups. Vaginal Infections and Prematurity Study Group." Am J Obstet Gynecol **174**(5): 1618-21.
Gomes, J. A., A. Romano, et al. (2005). "Amniotic membrane use in ophthalmology." Curr Opin Ophthalmol **16**(4): 233-40.
Gomez, R., R. Romero, et al. (1998). "The fetal inflammatory response syndrome." Am J Obstet Gynecol **179**(1): 194-202.
Goncalves, L. F., T. Chaiworapongsa, et al. (2002). "Intrauterine infection and prematurity." Ment Retard Dev Disabil Res Rev **8**(1): 3-13.
Gonzalez Bosquet, E., I. Ferrer, et al. (2005). "The value of interleukin-8, interleukin-6 and interleukin-1beta in vaginal wash as predictors of preterm delivery." Gynecol Obstet Invest **59**(3): 175-8.
Gravett, M. G., G. J. Haluska, et al. (1996). "Fetal and maternal endocrine responses to experimental intrauterine infection in rhesus monkeys." Am J Obstet Gynecol **174**(6): 1725-31; discussion 1731-3.
Gravett, M. G., S. S. Witkin, et al. (1994). "An experimental model for intraamniotic infection and preterm labor in rhesus monkeys." Am J Obstet Gynecol **171**(6): 1660-7.

Gray, D. J., H. B. Robinson, et al. (1992). "Adverse outcome in pregnancy following amniotic fluid isolation of Ureaplasma urealyticum." Prenat Diagn **12**(2): 111-7.

Grigsby, P. L., J. J. Hirst, et al. (2003). "Fetal responses to maternal and intra-amniotic lipopolysaccharide administration in sheep." Biol Reprod **68**(5): 1695-702.

Gross, G., T. Imamura, et al. (2000). "Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse." Am J Physiol Regul Integr Comp Physiol **278**(6): R1415-23.

Guaschino, S., F. De Seta, et al. (2006). "Aetiology of preterm labour: bacterial vaginosis." Bjog **113 Suppl 3**: 46-51.

Gunn, L., P. Hardiman, et al. (1996). "Measurement of interleukin-1 alpha and interleukin-6 in pregnancy-associated tissues." Reprod Fertil Dev **8**(7): 1069-73.

Haddad, J. J. (2002). "Cytokines and related receptor-mediated signaling pathways." Biochem Biophys Res Commun **297**(4): 700-13.

Haddad, R., G. Tromp, et al. (2006). "Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature." Am J Obstet Gynecol **195**(2): 394 e1-24.

Han, Y. W., R. W. Redline, et al. (2004). "Fusobacterium nucleatum induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth." Infect Immun **72**(4): 2272-9.

Hancock, R. E. and G. Diamond (2000). "The role of cationic antimicrobial peptides in innate host defences." Trends Microbiol **8**(9): 402-10.

Hansen, W. R., A. Drew, et al. (1999). "Regulation of cytosolic phospholipase A2 expression by cytokines in human amnion cells." Placenta **20**(4): 303-8.

Hao, H. N., J. Zhao, et al. (2001). "Induction of human beta-defensin-2 expression in human astrocytes by lipopolysaccharide and cytokines." J Neurochem **77**(4): 1027-35.

Harder, J., J. Bartels, et al. (1997). "A peptide antibiotic from human skin." Nature **387**(6636): 861.

Harder, J., J. Bartels, et al. (2001). "Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic." J Biol Chem **276**(8): 5707-13.

Harder, J., U. Meyer-Hoffert, et al. (2000). "Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia." Am J Respir Cell Mol Biol **22**(6): 714-21.

Harper, M. J. and R. C. Skarnes (1973). "The role of prostaglandin in endotoxin-induced abortion and fetal death." Adv Biosci **9**: 789-93.

Haruta, H., A. Kato, et al. (2001). "Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs." J Biol Chem **276**(16): 12485-8.

Hay, P. E., R. F. Lamont, et al. (1994). "Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage." Bmj **308**(6924): 295-8.

Hay, P. E., D. J. Morgan, et al. (1994). "A longitudinal study of bacterial vaginosis during pregnancy." Br J Obstet Gynaecol **101**(12): 1048-53.

Hayden, M. S. and S. Ghosh (2004). "Signaling to NF-kappaB." Genes Dev **18**(18): 2195-224.
Haynes, M. K., L. G. Jackson, et al. (1993). "Cytokine production in first trimester chorionic villi: detection of mRNAs and protein products in situ." Cell Immunol **151**(2): 300-8.
He, S. H., H. Xie, et al. (2004). "Inhibition of histamine release from human mast cells by natural chymase inhibitors." Acta Pharmacol Sin **25**(6): 822-6.
Hedges, S. R., F. Barrientes, et al. (2006). "Local and systemic cytokine levels in relation to changes in vaginal flora." J Infect Dis **193**(4): 556-62.
Heinig, J., S. Wilhelm, et al. (1993). "Semiquantitative determination of IL-1 alpha, TNF-alpha, PDGF-A, PDGF-B, and PDGF-receptor in term human placenta using polymerase chain reaction (PCR)." Zentralbl Gynakol **115**(7): 317-22.
Hellberg, D., S. Nilsson, et al. (2000). "Bacterial vaginosis and smoking." Int J STD AIDS **11**(9): 603-6.
Helmig, B. R., R. Romero, et al. (2002). "Neutrophil elastase and secretory leukocyte protease inhibitor in prelabor rupture of membranes, parturition and intra-amniotic infection." J Matern Fetal Neonatal Med **12**(4): 237-46.
Helmig, R., N. Uldbjerg, et al. (1995). "Secretory leukocyte protease inhibitor in the cervical mucus and in the fetal membranes." Eur J Obstet Gynecol Reprod Biol **59**(1): 95-101.
Henriksen, P. A., A. Devitt, et al. (2004). "Gene delivery of the elastase inhibitor elafin protects macrophages from neutrophil elastase-mediated impairment of apoptotic cell recognition." FEBS Lett **574**(1-3): 80-4.
Henriksen, P. A., M. Hitt, et al. (2004). "Adenoviral gene delivery of elafin and secretory leukocyte protease inhibitor attenuates NF-kappa B-dependent inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli." J Immunol **172**(7): 4535-44.
Hibbert, L. and J. A. Johnston (2001). "Cytokine signalling and disease." Expert Opin Ther Targets **5**(6): 641-653.
Hiemstra, P. S., R. J. Maassen, et al. (1996). "Antibacterial activity of antileukoprotease." Infect Immun **64**(11): 4520-4.
Hillier, S. L., M. A. Krohn, et al. (1992). "The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women." Obstet Gynecol **79**(3): 369-73.
Hillier, S. L., M. A. Krohn, et al. (1992). "Characteristics of three vaginal flora patterns assessed by Gram stain among pregnant women. Vaginal Infections and Prematurity Study Group." Am J Obstet Gynecol **166**(3): 938-44.
Hillier, S. L., R. P. Nugent, et al. (1995). "Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group." N Engl J Med **333**(26): 1737-42.
Hillier, S. L., S. S. Witkin, et al. (1993). "The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection." Obstet Gynecol **81**(6): 941-8.
Hirata, T., Y. Osuga, et al. (2007). "Expression of toll-like receptors 2, 3, 4, and 9 genes in the human endometrium during the menstrual cycle." J Reprod Immunol.**74**(1-2): 53-60

Hiratsuka, T., M. Nakazato, et al. (1998). "Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia." Biochem Biophys Res Commun **249**(3): 943-7.

Hirst, J. J., J. E. Mijovic, et al. (1998). "Prostaglandin endoperoxide H synthase-1 and -2 mRNA levels and enzyme activity in human decidua at term labor." J Soc Gynecol Investig **5**(1): 13-20.

Hitti, J., D. E. Riley, et al. (1997). "Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor." Clin Infect Dis **24**(6): 1228-32.

Hochstrasser, K., G. J. Albrecht, et al. (1981). "An elastase-specific inhibitor from human bronchial mucus. Isolation and characterization." Hoppe Seylers Z Physiol Chem **362**(10): 1369-75.

Holley, R. L., H. E. Richter, et al. (2004). "A randomized, double-blind clinical trial of vaginal acidification versus placebo for the treatment of symptomatic bacterial vaginosis." Sex Transm Dis **31**(4): 236-8.

Hollox, E. J., J. A. Armour, et al. (2003). "Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster." Am J Hum Genet **73**(3): 591-600.

Hollox, E. J., J. Davies, et al. (2005). "Beta-defensin genomic copy number is not a modifier locus for cystic fibrosis." J Negat Results Biomed **4**: 9.

Holmlund, U., G. Cebers, et al. (2002). "Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta." Immunology **107**(1): 145-51.

Horowitz, S., M. Mazor, et al. (1995). "Infection of the amniotic cavity with Ureaplasma urealyticum in the midtrimester of pregnancy." J Reprod Med **40**(5): 375-9.

Hsu, C. D., E. Meaddough, et al. (1998). "The role of amniotic fluid L-selectin, GRO-alpha, and interleukin-8 in the pathogenesis of intraamniotic infection." Am J Obstet Gynecol **178**(3): 428-32.

Hu, X. L., Y. Yang, et al. (1992). "Differential distribution of interleukin-1 alpha and interleukin-1 beta proteins in human placentas." J Reprod Immunol **22**(3): 257-68.

Huttner, K. M. and C. L. Bevins (1999). "Antimicrobial peptides as mediators of epithelial host defense." Pediatr Res **45**(6): 785-94.

Imahara, S. D. and G. E. O'Keefe (2004). "Genetic determinants of the inflammatory response." Curr Opin Crit Care **10**(5): 318-24.

Imseis, H. M., P. C. Greig, et al. (1997). "Characterization of the inflammatory cytokines in the vagina during pregnancy and labor and with bacterial vaginosis." J Soc Gynecol Investig **4**(2): 90-4.

Ito, A., T. Nakamura, et al. (1994). "Stimulation of the biosynthesis of interleukin 8 by interleukin 1 and tumor necrosis factor alpha in cultured human chorionic cells." Biol Pharm Bull **17**(11): 1463-7.

Ivell, R., T. Kimura, et al. (2001). "The structure and regulation of the oxytocin receptor." Exp Physiol **86**(2): 289-96.

Jaattela, M., P. Kuusela, et al. (1988). "Demonstration of tumor necrosis factor in human amniotic fluids and supernatants of placental and decidual tissues." Lab Invest **58**(1): 48-52.

Jalava, J., M. L. Mantymaa, et al. (1996). "Bacterial 16S rDNA polymerase chain reaction in the detection of intra-amniotic infection." Br J Obstet Gynaecol **103**(7): 664-9.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.

Jia, H. P., B. C. Schutte, et al. (2001). "Discovery of new human beta-defensins using a genomics-based approach." Gene **263**(1-2): 211-8.

Jin, F. Y., C. Nathan, et al. (1997). "Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide." Cell **88**(3): 417-26.

Joesoef, M. R., A. Karundeng, et al. (2001). "High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia." Contraception **64**(3): 169-72.

Jokhi, P. P., A. King, et al. (1997). "Cytokine production and cytokine receptor expression by cells of the human first trimester placental-uterine interface." Cytokine **9**(2): 126-37.

Joly, S., C. C. Organ, et al. (2005). "Correlation between beta-defensin expression and induction profiles in gingival keratinocytes." Mol Immunol **42**(9): 1073-84.

Jones, C. E. and K. Chan (2002). "Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells." Am J Respir Cell Mol Biol **26**(6): 748-53.

Jovanovic, D. V., J. A. Di Battista, et al. (1998). "IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages." J Immunol **160**(7): 3513-21.

Kaga, N., Y. Katsuki, et al. (1996). "Repeated administration of low-dose lipopolysaccharide induces preterm delivery in mice: a model for human preterm parturition and for assessment of the therapeutic ability of drugs against preterm delivery." Am J Obstet Gynecol **174**(2): 754-9.

Kajikawa, S., N. Kaga, et al. (1998). "Lipoteichoic acid induces preterm delivery in mice." J Pharmacol Toxicol Methods **39**(3): 147-54.

Kalinka, J., W. Sobala, et al. (2005). "Decreased proinflammatory cytokines in cervicovaginal fluid, as measured in midgestation, are associated with preterm delivery." Am J Reprod Immunol **54**(2): 70-6.

Kalkhoven, E., S. Wissink, et al. (1996). "Negative interaction between the RelA(p65) subunit of NF-kappaB and the progesterone receptor." J Biol Chem **271**(11): 6217-24.

Kanda, N., S. Koike, et al. (2005). "IL-17 suppresses TNF-alpha-induced CCL27 production through induction of COX-2 in human keratinocytes." J Allergy Clin Immunol **116**(5): 1144-50.

Kao, C. Y., Y. Chen, et al. (2004). "IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways." J Immunol **173**(5): 3482-91.

Karin, M. and M. Delhase (2000). "The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling." Semin Immunol **12**(1): 85-98.

Karin, M., Z. Liu, et al. (1997). "AP-1 function and regulation." Curr Opin Cell Biol **9**(2): 240-6.

Katsuki, Y., N. Kaga, et al. (1997). "Ability of intrauterine bacterial lipopolysaccharide to cause in situ uterine contractions in pregnant rabbits." Acta Obstet Gynecol Scand **76**(1): 26-32.
Kauma, S., D. Matt, et al. (1990). "Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes." Am J Obstet Gynecol **163**(5 Pt 1): 1430-7.
Kawaguchi, M., M. Adachi, et al. (2004). "IL-17 cytokine family." J Allergy Clin Immunol **114**(6): 1265-73; quiz 1274.
Keane, F. E., R. Maw, et al. (2005). "Methods employed by genitourinary medicine clinics in the United Kingdom to diagnose bacterial vaginosis." Sex Transm Infect **81**(2): 155-7.
Keelan, J., R. Helliwell, et al. (2001). "15-deoxy-delta12,14-prostaglandin J2-induced apoptosis in amnion-like WISH cells." Prostaglandins Other Lipid Mediat **66**(4): 265-82.
Keelan, J. A., M. Blumenstein, et al. (2003). "Cytokines, prostaglandins and parturition--a review." Placenta **24 Suppl A**: S33-46.
Keelan, J. A., K. W. Marvin, et al. (1999). "Cytokine abundance in placental tissues: evidence of inflammatory activation in gestational membranes with term and preterm parturition." Am J Obstet Gynecol **181**(6): 1530-6.
Keelan, J. A., T. Sato, et al. (1997). "Interleukin (IL)-6 and IL-8 production by human amnion: regulation by cytokines, growth factors, glucocorticoids, phorbol esters, and bacterial lipopolysaccharide." Biol Reprod **57**(6): 1438-44.
Kelly, R. W. (1994). "Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response." Endocr Rev **15**(5): 684-706.
Kelly, R. W. (1996). "Inflammatory mediators and parturition." Rev Reprod **1**(2): 89-96.
Kelly, R. W. (2002). "Inflammatory mediators and cervical ripening." J Reprod Immunol **57**(1-2): 217-24.
Kelly, R. W., G. G. Carr, et al. (1995). "Prostaglandin and cytokine release by trophoblastic villi." Hum Reprod **10**(12): 3289-92.
Kenyon, S., M. Boulvain, et al. (2004). "Antibiotics for preterm rupture of the membranes: a systematic review." Obstet Gynecol **104**(5 Pt 1): 1051-7.
Kenyon, S. L., D. J. Taylor, et al. (2001). "Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group." Lancet **357**(9261): 979-88.
Kenyon, S. L., D. J. Taylor, et al. (2001). "Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group." Lancet **357**(9261): 989-94.
Khabar, K. S., F. Al-Zoghaibi, et al. (1997). "Interleukin-8 selectively enhances cytopathic effect (CPE) induced by positive-strand RNA viruses in the human WISH cell line." Biochem Biophys Res Commun **235**(3): 774-8.
Kikuchi, T., T. Abe, et al. (1997). "Cis-acting region associated with lung cell-specific expression of the secretory leukoprotease inhibitor gene." Am J Respir Cell Mol Biol **17**(3): 361-7.

Kim, G. J., R. Romero, et al. (2005). "Expression of bone morphogenetic protein 2 in normal spontaneous labor at term, preterm labor, and preterm premature rupture of membranes." Am J Obstet Gynecol **193**(3 Pt 2): 1137-43.
Kim, Y. M., R. Romero, et al. (2004). "Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis." Am J Obstet Gynecol **191**(4): 1346-55.
Kim, Y. M., R. Romero, et al. (2005). "Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia?" Am J Obstet Gynecol **193**(3 Pt 2): 921-7.
King, A., P. P. Jokhi, et al. (1995). "Screening for cytokine mRNA in human villous and extravillous trophoblasts using the reverse-transcriptase polymerase chain reaction (RT-PCR)." Cytokine **7**(4): 364-71.
King, A. E., H. O. Critchley, et al. (2000). "Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role." Mol Hum Reprod **6**(2): 191-6.
King, A. E., H. O. Critchley, et al. (2003). "Innate immune defences in the human endometrium." Reprod Biol Endocrinol **1**(1): 116.
King, A. E., H. O. Critchley, et al. (2003). "Elafin in human endometrium: an antiprotease and antimicrobial molecule expressed during menstruation." J Clin Endocrinol Metab **88**(9): 4426-31.
King, A. E., D. C. Fleming, et al. (2002). "Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells." Mol Hum Reprod **8**(4): 341-9.
King, A. E., D. C. Fleming, et al. (2003). "Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human endometrium." J Reprod Immunol **59**(1): 1-16.
King, A. E., K. Morgan, et al. (2003). "Differential regulation of secretory leukocyte protease inhibitor and elafin by progesterone." Biochem Biophys Res Commun **310**(2): 594-9.
King, A. E., A. Paltoo, et al. (2007). "Expression of natural antimicrobials by human placenta and fetal membranes." Placenta **28**(2-3): 161-9.
King, J. and V. Flenady (2002). "Prophylactic antibiotics for inhibiting preterm labour with intact membranes." Cochrane Database Syst Rev(4): CD000246.
Kjaergaard, N., M. Hein, et al. (2001). "Antibacterial properties of human amnion and chorion in vitro." Eur J Obstet Gynecol Reprod Biol **94**(2): 224-9.
Kjaergaard, N., R. B. Helmig, et al. (1999). "Chorioamniotic membranes constitute a competent barrier to group b streptococcus in vitro." Eur J Obstet Gynecol Reprod Biol **83**(2): 165-9.
Klebanoff, M. and K. Searle (2006). "The role of inflammation in preterm birth-focus on periodontitis." Bjog **113 Suppl 3**: 43-5.
Klebanoff, M. A., S. L. Hillier, et al. (2005). "Is bacterial vaginosis a stronger risk factor for preterm birth when it is diagnosed earlier in gestation?" Am J Obstet Gynecol **192**(2): 470-7.
Kniss, D. A., Y. Xie, et al. (2002). "ED(27) trophoblast-like cells isolated from first-trimester chorionic villi are genetically identical to HeLa cells yet exhibit a distinct phenotype." Placenta **23**(1): 32-43.

Kolls, J. K. and A. Linden (2004). "Interleukin-17 family members and inflammation." Immunity **21**(4): 467-76.
Kracht, M. and J. Saklatvala (2002). "Transcriptional and post-transcriptional control of gene expression in inflammation." Cytokine **20**(3): 91-106.
Kramps, J. A., C. Franken, et al. (1984). "ELISA for quantitative measurement of low-molecular-weight bronchial protease inhibitor in human sputum." Am Rev Respir Dis **129**(6): 959-63.
Krisanaprakornkit, S., J. R. Kimball, et al. (2000). "Inducible expression of human beta-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier." Infect Immun **68**(5): 2907-15.
Krisanaprakornkit, S., A. Weinberg, et al. (1998). "Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue." Infect Immun **66**(9): 4222-8.
Kumazaki, K., M. Nakayama, et al. (2004). "Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis." Hum Pathol **35**(1): 47-54.
Kurki, T., A. Sivonen, et al. (1992). "Bacterial vaginosis in early pregnancy and pregnancy outcome." Obstet Gynecol **80**(2): 173-7.
Kyriakis, J. M. and J. Avruch (2001). "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation." Physiol Rev **81**(2): 807-69.
Laham, N., S. P. Brennecke, et al. (1994). "Tumour necrosis factor alpha during human pregnancy and labour: maternal plasma and amniotic fluid concentrations and release from intrauterine tissues." Eur J Endocrinol **131**(6): 607-14.
Laham, N., S. P. Brennecke, et al. (1996). "Labour-associated increase in interleukin-1 alpha release in vitro by human gestational tissues." J Endocrinol **150**(3): 515-22.
Laham, N., S. P. Brennecke, et al. (1997). "Interleukin-8 release from human gestational tissue explants: the effects of lipopolysaccharide and cytokines." Biol Reprod **57**(3): 616-20.
Laham, N., S. P. Brennecke, et al. (1999). "Interleukin-8 release from human gestational tissue explants: effects of gestation, labor, and chorioamnionitis." Biol Reprod **61**(3): 823-7.
Laham, N., G. E. Rice, et al. (1993). "Interleukin 8 concentrations in amniotic fluid and peripheral venous plasma during human pregnancy and parturition." Acta Endocrinol (Copenh) **129**(3): 220-4.
Lappas, M., M. Permezel, et al. (2002). "Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro." Biol Reprod **67**(2): 668-73.
Lappas, M., M. Permezel, et al. (2003). "N-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro." J Clin Endocrinol Metab **88**(4): 1723-9.

Lee, P. R., S. R. Kim, et al. (2003). "Therapeutic effect of cyclo-oxygenase inhibitors with different isoform selectivity in lipopolysaccharide-induced preterm birth in mice." Am J Obstet Gynecol **189**(1): 261-6.
Lefebvre, D. L., M. Piersanti, et al. (1995). "Myometrial transcriptional regulation of the gap junction gene, connexin-43." Reprod Fertil Dev **7**(3): 603-11.
Lehrer, R. I. (2004). "Primate defensins." Nat Rev Microbiol **2**(9): 727-38.
Lei, H., E. E. Furth, et al. (1996). "A program of cell death and extracellular matrix degradation is activated in the amnion before the onset of labor." J Clin Invest **98**(9): 1971-8.
Leitich, H., B. Bodner-Adler, et al. (2003). "Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis." Am J Obstet Gynecol **189**(1): 139-47.
Lentsch, A. B., J. A. Jordan, et al. (1999). "Inhibition of NF-kappaB activation and augmentation of IkappaBbeta by secretory leukocyte protease inhibitor during lung inflammation." Am J Pathol **154**(1): 239-47.
Librach, C. L., S. L. Feigenbaum, et al. (1994). "Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro." J Biol Chem **269**(25): 17125-31.
Liggins, G. C. (1989). "Initiation of labour." Biol Neonate **55**(6): 366-75.
Lindstrom, T. M. and P. R. Bennett (2005). "15-Deoxy-{Delta}12,14-Prostaglandin J2 inhibits IL-1{beta}-induced NF-{kappa}B in human amnion and myometrial cells: Mechanisms and implications." J Clin Endocrinol Metab. **90**(6); 3534-43
Lindstrom, T. M. and P. R. Bennett (2005). "The role of nuclear factor kappa B in human labour." Reproduction **130**(5): 569-81.
Liu, L., L. Wang, et al. (1998). "Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation." Gene **222**(2): 237-44.
Locksley, R. M., N. Killeen, et al. (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology." Cell **104**(4): 487-501.
Lonsdale, L. B., M. G. Elder, et al. (1996). "A comparison of cytokine and hormone production by decidual cells and tissue explants." J Endocrinol **151**(2): 309-13.
Lorenz, E., M. Hallman, et al. (2002). "Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population." Pediatr Res **52**(3): 373-6.
Lukiw, W. J., R. P. Pelaez, et al. (1998). "Budesonide epimer R or dexamethasone selectively inhibit platelet-activating factor-induced or interleukin 1beta-induced DNA binding activity of cis-acting transcription factors and cyclooxygenase-2 gene expression in human epidermal keratinocytes." Proc Natl Acad Sci U S A **95**(7): 3914-9.
Macones, G. A., S. Parry, et al. (2004). "A polymorphism in the promoter region of TNF and bacterial vaginosis: preliminary evidence of gene-environment interaction in the etiology of spontaneous preterm birth." Am J Obstet Gynecol **190**(6): 1504-8; discussion 3A.
Mahendroo, M. S., A. Porter, et al. (1999). "The parturition defect in steroid 5alpha-reductase type 1 knockout mice is due to impaired cervical ripening." Mol Endocrinol **13**(6): 981-92.

Malak, T. M., C. D. Ockleford, et al. (1993). "Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes." Placenta **14**(4): 385-406.
Maruyama, M., J. G. Hay, et al. (1994). "Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester." J Clin Invest **94**(1): 368-75.
Marvin, K. W., J. A. Keelan, et al. (2002). "Use of cDNA arrays to generate differential expression profiles for inflammatory genes in human gestational membranes delivered at term and preterm." Mol Hum Reprod **8**(4): 399-408.
Massova, I., L. P. Kotra, et al. (1998). "Matrix metalloproteinases: structures, evolution, and diversification." Faseb J **12**(12): 1075-95.
Mathews, M., H. P. Jia, et al. (1999). "Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands." Infect Immun **67**(6): 2740-5.
Matsukawa, A., C. M. Hogaboam, et al. (2000). "Chemokines and innate immunity." Rev Immunogenet **2**(3): 339-58.
Matsuzaki, K. (1999). "Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes." Biochim Biophys Acta **1462**(1-2): 1-10.
Mattsby-Baltzer, I., J. J. Platz-Christensen, et al. (1998). "IL-1beta, IL-6, TNFalpha, fetal fibronectin, and endotoxin in the lower genital tract of pregnant women with bacterial vaginosis." Acta Obstet Gynecol Scand **77**(7): 701-6.
McCann, M. F., D. E. Irwin, et al. (1992). "Nicotine and cotinine in the cervical mucus of smokers, passive smokers, and nonsmokers." Cancer Epidemiol Biomarkers Prev **1**(2): 125-9.
McDermott, A. M., R. L. Redfern, et al. (2003). "Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells." Invest Ophthalmol Vis Sci **44**(5): 1859-65.
McDonald, H., P. Brocklehurst, et al. (2007). "Antibiotics for treating bacterial vaginosis in pregnancy." Cochrane Database Syst Rev(1): CD000262.
McGregor, J. A., J. I. French, et al. (1995). "Prevention of premature birth by screening and treatment for common genital tract infections: results of a prospective controlled evaluation." Am J Obstet Gynecol **173**(1): 157-67.
McGregor, J. A., J. I. French, et al. (1990). "Antenatal microbiologic and maternal risk factors associated with prematurity." Am J Obstet Gynecol **163**(5 Pt 1): 1465-73.
McLaren, J., D. J. Taylor, et al. (2000). "Prostaglandin E(2)-dependent production of latent matrix metalloproteinase-9 in cultures of human fetal membranes." Mol Hum Reprod **6**(11): 1033-40.
McLean, M., A. Bisits, et al. (1995). "A placental clock controlling the length of human pregnancy." Nat Med **1**(5): 460-3.
McMichael, J. W., A. I. Maxwell, et al. (2005). "Antimicrobial activity of murine lung cells against Staphylococcus aureus is increased in vitro and in vivo after elafin gene transfer." Infect Immun **73**(6): 3609-17.
McMichael, J. W., A. Roghanian, et al. (2005). "The antimicrobial antiproteinase elafin binds to lipopolysaccharide and modulates macrophage responses." Am J Respir Cell Mol Biol **32**(5): 443-52.

McNeely, T. B., M. Dealy, et al. (1995). "Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro." J Clin Invest **96**(1): 456-64.
McNeely, T. B., D. C. Shugars, et al. (1997). "Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription." Blood **90**(3): 1141-9.
Medzhitov, R. and C. Janeway, Jr. (2000). "The Toll receptor family and microbial recognition." Trends Microbiol **8**(10): 452-6.
Medzhitov, R., P. Preston-Hurlburt, et al. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." Nature **388**(6640): 394-7.
Menon, R. and S. J. Fortunato (2004). "The role of matrix degrading enzymes and apoptosis in rupture of membranes." J Soc Gynecol Investig **11**(7): 427-37.
Menon, R., S. J. Lombardi, et al. (2002). "TNF-alpha promotes caspase activation and apoptosis in human fetal membranes." J Assist Reprod Genet **19**(4): 201-4.
Menon, R., K. F. Swan, et al. (1995). "Expression of inflammatory cytokines (interleukin-1 beta and interleukin-6) in amniochorionic membranes." Am J Obstet Gynecol **172**(2 Pt 1): 493-500.
Menon, R., D. R. Velez, et al. (2006). "Multilocus interactions at maternal tumor necrosis factor-alpha, tumor necrosis factor receptors, interleukin-6 and interleukin-6 receptor genes predict spontaneous preterm labor in European-American women." Am J Obstet Gynecol **194**(6): 1616-24.
Meyer-Hoffert, U., N. Wichmann, et al. (2003). "Supernatants of Pseudomonas aeruginosa induce the Pseudomonas-specific antibiotic elafin in human keratinocytes." Exp Dermatol **12**(4): 418-25.
Miggin, S. M. and L. A. O'Neill (2006). "New insights into the regulation of TLR signaling." J Leukoc Biol **80**(2): 220-6.
Mijovic, J. E., T. Zakar, et al. (1999). "Prostaglandin endoperoxide H synthase mRNA expression in the human amnion and decidua during pregnancy and in the amnion at preterm labour." Mol Hum Reprod **5**(2): 182-7.
Mijovic, J. E., T. Zakar, et al. (1997). "Prostaglandin-endoperoxide H synthase-2 expression and activity increases with term labor in human chorion." Am J Physiol **272**(5 Pt 1): E832-40.
Miki, T., T. Lehmann, et al. (2005). "Stem cell characteristics of amniotic epithelial cells." Stem Cells **23**(10): 1549-59.
Milani, M., E. Barcellona, et al. (2003). "Efficacy of the combination of 2 g oral tinidazole and acidic buffering vaginal gel in comparison with vaginal clindamycin alone in bacterial vaginosis: a randomized, investigator-blinded, controlled trial." Eur J Obstet Gynecol Reprod Biol **109**(1): 67-71.
Miller, J., J. Michel, et al. (1976). "Studies on the antimicrobial activity of amniotic fluid." Am J Obstet Gynecol **125**(2): 212-4.
Milner, S. M., A. Cole, et al. (2003). "Inducibility of HBD-2 in acute burns and chronic conditions of the lung." Burns **29**(6): 553-5.
Mitchell, M. D. (1991). "Endothelins in perinatal biology." Semin Perinatol **15**(1): 79-85.

Mitchell, M. D., S. S. Edwin, et al. (1993). "Mechanism of interleukin-1 beta stimulation of human amnion prostaglandin biosynthesis: mediation via a novel inducible cyclooxygenase." Placenta **14**(6): 615-25.
Mitchell, M. D., R. J. Romero, et al. (1990). "Actions of endothelin-1 on prostaglandin production by gestational tissues." Prostaglandins **40**(6): 627-35.
Mitsuhashi, H., S. Asano, et al. (1996). "Administration of truncated secretory leukoprotease inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters." Am J Respir Crit Care Med **153**(1): 369-74.
Mohan, A. R., S. R. Sooranna, et al. (2007). "The Effect Of Mechanical Stretch On Cyclo-Oxygenase Type 2 Expression And AP-1 And NF-{Kappa}B Activity In Human Amnion Cells." Endocrinology. **148**(4): 1850-7
Morris, M. C., P. A. Rogers, et al. (2001). "Is bacterial vaginosis a sexually transmitted infection?" Sex Transm Infect **77**(1): 63-8.
Moseley, T. A., D. R. Haudenschild, et al. (2003). "Interleukin-17 family and IL-17 receptors." Cytokine Growth Factor Rev **14**(2): 155-74.
Motoyama, M., S. Yamazaki, et al. (2005). "Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein." J Biol Chem **280**(9): 7444-51.
Murata, E., S. Sharmin, et al. (2003). "The effect of topically applied secretory leukocyte protease inhibitor on the eosinophil response in the late phase of allergic conjunctivitis." Curr Eye Res **26**(5): 271-6.
Mussalli, G. M., R. Blanchard, et al. (1999). "Inflammatory cytokines in a murine model of infection-induced preterm labor: cause or effect?" J Soc Gynecol Investig **6**(4): 188-95.
Nagase, H. and J. F. Woessner, Jr. (1999). "Matrix metalloproteinases." J Biol Chem **274**(31): 21491-4.
Nara, K., S. Ito, et al. (1994). "Elastase inhibitor elafin is a new type of proteinase inhibitor which has a transglutaminase-mediated anchoring sequence termed "cementoin"." J Biochem (Tokyo) **115**(3): 441-8.
Nelson-Rees, W. A. and R. R. Flandermeyer (1976). "HeLa cultures defined." Science **191**(4222): 96-8.
Ness, R. B., S. L. Hillier, et al. (2002). "Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina." Obstet Gynecol **100**(4): 765.
Nitschke, M., S. Wiehl, et al. (2002). "Bactericidal activity of renal tubular cells: the putative role of human beta-defensins." Exp Nephrol **10**(5-6): 332-7.
Niyonsaba, F., M. Hirata, et al. (2003). "Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: multifunctional activities on mast cells." Curr Drug Targets Inflamm Allergy **2**(3): 224-31.
Niyonsaba, F., K. Iwabuchi, et al. (2002). "Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway." Int Immunol **14**(4): 421-6.
Niyonsaba, F., H. Ogawa, et al. (2004). "Human beta-defensin-2 functions as a chemotactic agent for tumour necrosis factor-alpha-treated human neutrophils." Immunology **111**(3): 273-81.

Niyonsaba, F., H. Ushio, et al. (2005). "The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes." J Immunol **175**(3): 1776-84.
Niyonsaba, F., H. Ushio, et al. (2007). "Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines." J Invest Dermatol **127**(3): 594-604.
Nugent, R. P., M. A. Krohn, et al. (1991). "Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation." J Clin Microbiol **29**(2): 297-301.
O'Neil, D. A., E. M. Porter, et al. (1999). "Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium." J Immunol **163**(12): 6718-24.
O'Neill, L. (2000). "The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence." Biochem Soc Trans **28**(5): 557-63.
Odaka, C., T. Mizuochi, et al. (2003). "Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response." J Immunol **171**(3): 1507-14.
Ogura, H., M. Yoshinouchi, et al. (1993). "Human papillomavirus type 18 DNA in so-called HEP-2, KB and FL cells--further evidence that these cells are HeLa cell derivatives." Cell Mol Biol (Noisy-le-grand) **39**(5): 463-7.
Ohta, K., T. Nakajima, et al. (2004). "Elafin-overexpressing mice have improved cardiac function after myocardial infarction." Am J Physiol Heart Circ Physiol **287**(1): H286-92.
Olmsted, S. S., L. A. Meyn, et al. (2003). "Glycosidase and proteinase activity of anaerobic Gram-negative bacteria isolated from women with bacterial vaginosis." Sex Transm Dis **30**(3): 257-61.
Olson, D. M. (2003). "The role of prostaglandins in the initiation of parturition." Best Pract Res Clin Obstet Gynaecol **17**(5): 717-30.
Osman, I., A. Young, et al. (2003). "Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term." Mol Hum Reprod **9**(1): 41-5.
Ostojic, S., S. Dubanchet, et al. (2003). "Demonstration of the presence of IL-16, IL-17 and IL-18 at the murine fetomaternal interface during murine pregnancy." Am J Reprod Immunol **49**(2): 101-12.
Palsson-McDermott, E. M. and L. A. O'Neill (2004). "Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4." Immunology **113**(2): 153-62.
Panter, K. R., M. E. Hannah, et al. (1999). "The effect of indomethacin tocolysis in preterm labour on perinatal outcome: a randomised placebo-controlled trial." Br J Obstet Gynaecol **106**(5): 467-73.
Paradowska, E., Z. Blach-Olszewska, et al. (1997). "Constitutive and induced cytokine production by human placenta and amniotic membrane at term." Placenta **18**(5-6): 441-6.
Paulesu, L., A. King, et al. (1991). "Immunohistochemical localization of IL-1 alpha and IL-1 beta in normal human placenta." Lymphokine Cytokine Res **10**(6): 443-8.

Peebles, D. M. and J. S. Wyatt (2002). "Synergy between antenatal exposure to infection and intrapartum events in causation of perinatal brain injury at term." Bjog **109**(7): 737-9.
Pfundt, R., M. Wingens, et al. (2000). "TNF-alpha and serum induce SKALP/elafin gene expression in human keratinocytes by a p38 MAP kinase-dependent pathway." Arch Dermatol Res **292**(4): 180-7.
Pieber, D., V. C. Allport, et al. (2001). "Interactions between progesterone receptor isoforms in myometrial cells in human labour." Mol Hum Reprod **7**(9): 875-9.
Pijnenborg, R., P. J. McLaughlin, et al. (1998). "Immunolocalization of tumour necrosis factor-alpha (TNF-alpha) in the placental bed of normotensive and hypertensive human pregnancies." Placenta **19**(4): 231-9.
Pillay, K., A. Coutsoudis, et al. (2001). "Secretory leukocyte protease inhibitor in vaginal fluids and perinatal human immunodeficiency virus type 1 transmission." J Infect Dis **183**(4): 653-6.
Pioli, P. A., E. Amiel, et al. (2004). "Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract." Infect Immun **72**(10): 5799-806.
Pivarcsi, A., I. Nagy, et al. (2005). "Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human β-defensin-2 in vaginal epithelial cells." Microbes and Infection **7**(9-10): 1117.
Pivarcsi, A., I. Nagy, et al. (2005). "Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human beta-defensin-2 in vaginal epithelial cells." Microbes Infect **7**(9-10): 1117-27.
Platz-Christensen, J. J., I. Mattsby-Baltzer, et al. (1993). "Endotoxin and interleukin-1 alpha in the cervical mucus and vaginal fluid of pregnant women with bacterial vaginosis." Am J Obstet Gynecol **169**(5): 1161-6.
Poli, V. (1998). "The role of C/EBP isoforms in the control of inflammatory and native immunity functions." J Biol Chem **273**(45): 29279-82.
Pongcharoen, S., J. Somran, et al. (2007). "Interleukin-17 expression in the human placenta." Placenta **28**(1): 59-63.
Quayle, A. J., E. M. Porter, et al. (1998). "Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract." Am J Pathol **152**(5): 1247-58.
Ramsey, P. S., M. D. Lyon, et al. (2005). "Use of vaginal polymorphonuclear to epithelial cell ratios for the prediction of preterm birth." Obstet Gynecol **105**(1): 139-44.
Ravishanker, R., A. S. Bath, et al. (2003). ""Amnion Bank"--the use of long term glycerol preserved amniotic membranes in the management of superficial and superficial partial thickness burns." Burns **29**(4): 369-74.
Reznikov, L. L., G. Fantuzzi, et al. (1999). "Utilization of endoscopic inoculation in a mouse model of intrauterine infection-induced preterm birth: role of interleukin 1beta." Biol Reprod **60**(5): 1231-8.
Rice, W. G., T. Ganz, et al. (1987). "Defensin-rich dense granules of human neutrophils." Blood **70**(3): 757-65.
Riley, S. C., R. Leask, et al. (1999). "Secretion of tissue inhibitors of matrix metalloproteinases by human fetal membranes, decidua and placenta at parturition." J Endocrinol **162**(3): 351-9.

Riley, S. C., J. C. Walton, et al. (1991). "The localization and distribution of corticotropin-releasing hormone in the human placenta and fetal membranes throughout gestation." J Clin Endocrinol Metab **72**(5): 1001-7.
Rindsjo, E., U. Holmlund, et al. (2007). "Toll-like receptor-2 expression in normal and pathologic human placenta." Hum Pathol.
Roghanian, A., S. E. Williams, et al. (2006). "The antimicrobial/elastase inhibitor elafin regulates lung dendritic cells and adaptive immunity." Am J Respir Cell Mol Biol **34**(5): 634-42.
Romero, R., M. Ceska, et al. (1991). "Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition." Am J Obstet Gynecol **165**(4 Pt 1): 813-20.
Romero, R., J. Espinoza, et al. (2002). "Infection and prematurity and the role of preventive strategies." Semin Neonatol **7**(4): 259-74.
Romero, R., J. Espinoza, et al. (2006). "Inflammation in preterm and term labour and delivery." Semin Fetal Neonatal Med **11**(5): 317-26.
Romero, R., J. Espinoza, et al. (2006). "The preterm parturition syndrome." Bjog **113 Suppl 3**: 17-42.
Romero, R., K. R. Manogue, et al. (1989). "Infection and labor. IV. Cachectin-tumor necrosis factor in the amniotic fluid of women with intraamniotic infection and preterm labor." Am J Obstet Gynecol **161**(2): 336-41.
Romero, R., M. Mazor, et al. (1992). "Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition." Am J Reprod Immunol **27**(3-4): 117-23.
Romero, R., M. Mazor, et al. (1992). "Tumor necrosis factor in preterm and term labor." Am J Obstet Gynecol **166**(5): 1576-87.
Romero, R., M. Mazor, et al. (1991). "Systemic administration of interleukin-1 induces preterm parturition in mice." Am J Obstet Gynecol **165**(4 Pt 1): 969-71.
Romero, R., S. T. Parvizi, et al. (1990). "Amniotic fluid interleukin-1 in spontaneous labor at term." J Reprod Med **35**(3): 235-8.
Romero, R., M. Sirtori, et al. (1989). "Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes." Am J Obstet Gynecol **161**(3): 817-24.
Romero, R., Y. K. Wu, et al. (1989). "Human decidua: a source of interleukin-1." Obstet Gynecol **73**(1): 31-4.
Roos, T., T. R. Martin, et al. (1997). "Lipopolysaccharide binding protein and soluble CD14 receptor protein in amniotic fluid and cord blood in patients at term." Am J Obstet Gynecol **177**(5): 1230-7.
Rosenstein, I. J., D. J. Morgan, et al. (1996). "Bacterial vaginosis in pregnancy: distribution of bacterial species in different Gram-stain categories of the vaginal flora." J Med Microbiol **45**(2): 120-6.
Ross, J. (1995). "mRNA stability in mammalian cells." Microbiol Rev **59**(3): 423-50.
Royce, R. A., T. P. Jackson, et al. (1999). "Race/ethnicity, vaginal flora patterns, and pH during pregnancy." Sex Transm Dis **26**(2): 96-102.
Sachs, B. P. and C. M. Stern (1979). "Activity and characterization of a low molecular fraction present in human amniotic fluid with broad spectrum antibacterial activity." Br J Obstet Gynaecol **86**(2): 81-6.

Sadovsky, Y., D. M. Nelson, et al. (2000). "Effective diminution of amniotic prostaglandin production by selective inhibitors of cyclooxygenase type 2." Am J Obstet Gynecol **182**(2): 370-6.

Sadowsky, D. W., K. M. Adams, et al. (2006). "Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model." Am J Obstet Gynecol **195**(6): 1578-89.

Saito, S., T. Kasahara, et al. (1993). "Elevation of amniotic fluid interleukin 6 (IL-6), IL-8 and granulocyte colony stimulating factor (G-CSF) in term and preterm parturition." Cytokine **5**(1): 81-8.

Saito, S., T. Kasahara, et al. (1994). "Interleukin-8 production by CD16-CD56bright natural killer cells in the human early pregnancy decidua." Biochem Biophys Res Commun **200**(1): 378-83.

Saito, S., T. Kasahara, et al. (1994). "Detection and localization of interleukin-8 mRNA and protein in human placenta and decidual tissues." J Reprod Immunol **27**(3): 161-72.

Saito, S., K. Nishikawa, et al. (1993). "Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua." Int Immunol **5**(5): 559-63.

Saitoh, H., T. Masuda, et al. (2001). "Secretion and gene expression of secretory leukocyte protease inhibitor by human airway submucosal glands." Am J Physiol Lung Cell Mol Physiol **280**(1): L79-87.

Sallenave, J. M. (2002). "Antimicrobial activity of antiproteinases." Biochem Soc Trans **30**(2): 111-5.

Sallenave, J. M., G. A. Cunningham, et al. (2003). "Regulation of pulmonary and systemic bacterial lipopolysaccharide responses in transgenic mice expressing human elafin." Infect Immun **71**(7): 3766-74.

Sallenave, J. M. and A. P. Ryle (1991). "Purification and characterization of elastase-specific inhibitor. Sequence homology with mucus proteinase inhibitor." Biol Chem Hoppe Seyler **372**(1): 13-21.

Sallenave, J. M., J. Shulmann, et al. (1994). "Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes." Am J Respir Cell Mol Biol **11**(6): 733-41.

Sallenave, J. M., M. Si Tahar, et al. (1997). "Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human neutrophils." J Leukoc Biol **61**(6): 695-702.

Sallenave, J. M. and A. Silva (1993). "Characterization and gene sequence of the precursor of elafin, an elastase-specific inhibitor in bronchial secretions." Am J Respir Cell Mol Biol **8**(4): 439-45.

Sallenave, J. M., A. Silva, et al. (1993). "Secretion of mucus proteinase inhibitor and elafin by Clara cell and type II pneumocyte cell lines." Am J Respir Cell Mol Biol **8**(2): 126-33.

Sancho, D., M. Gomez, et al. (2005). "CD69 is an immunoregulatory molecule induced following activation." Trends Immunol **26**(3): 136-40.

Sancho, D., M. Gomez, et al. (2003). "CD69 downregulates autoimmune reactivity through active transforming growth factor-beta production in collagen-induced arthritis." J Clin Invest **112**(6): 872-82.

Sano, C., T. Shimizu, et al. (2000). "Effects of secretory leucocyte protease inhibitor on the production of the anti-inflammatory cytokines, IL-10 and transforming growth factor-beta (TGF-beta), by lipopolysaccharide-stimulated macrophages." Clin Exp Immunol **121**(1): 77-85.
Sano, C., T. Shimizu, et al. (2003). "Effects of secretory leukocyte protease inhibitor on the tumor necrosis factor-alpha production and NF-kappaB activation of lipopolysaccharide-stimulated macrophages." Cytokine **21**(1): 38-42.
Sato, T. A., J. A. Keelan, et al. (2002). "Efficacy and specificity of non-steroidal anti-inflammatory drugs for the inhibition of cytokine-stimulated prostaglandin E(2) secretion by amnion-derived WISH cells." Prostaglandins Leukot Essent Fatty Acids **66**(5-6): 525-7.
Sawdy, R., H. Pan, et al. (2003). "Effect of selective vs. non-selective cyclo-oxygenase inhibitors on fetal membrane prostaglandin synthesis." J Obstet Gynaecol **23**(3): 239-43.
Sawdy, R. J., D. M. Slater, et al. (2000). "The roles of the cyclo-oxygenases types one and two in prostaglandin synthesis in human fetal membranes at term." Placenta **21**(1): 54-7.
Schalkwijk, J., O. Wiedow, et al. (1999). "The trappin gene family: proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core." Biochem J **340 (Pt 3)**: 569-77.
Scheetz, T., J. A. Bartlett, et al. (2002). "Genomics-based approaches to gene discovery in innate immunity." Immunol Rev **190**: 137-45.
Scheidereit, C. (2006). "IkappaB kinase complexes: gateways to NF-kappaB activation and transcription." Oncogene **25**(51): 6685-705.
Schlafer, D. H., B. Yuh, et al. (1994). "Effect of Salmonella endotoxin administered to the pregnant sheep at 133-142 days gestation on fetal oxygenation, maternal and fetal adrenocorticotropic hormone and cortisol, and maternal plasma tumor necrosis factor alpha concentrations." Biol Reprod **50**(6): 1297-302.
Schmid, M., K. Fellermann, et al. (2007). "Attenuated induction of epithelial and leukocyte serine antiproteases elafin and secretory leukocyte protease inhibitor in Crohn's disease." J Leukoc Biol. **81**(4): 907-15
Schmidt, H. and J. G. Hansen (2001). "Validity of wet-mount bacterial morphotype identification of vaginal fluid by phase-contrast microscopy for diagnosis of bacterial vaginosis in family practice." Apmis **109**(9): 589-94.
Schonwetter, B. S., E. D. Stolzenberg, et al. (1995). "Epithelial antibiotics induced at sites of inflammation." Science **267**(5204): 1645-8.
Schroder, J. M. (1999). "Epithelial antimicrobial peptides: innate local host response elements." Cell Mol Life Sci **56**(1-2): 32-46.
Schroeder, A., O. Mueller, et al. (2006). "The RIN: an RNA integrity number for assigning integrity values to RNA measurements." BMC Mol Biol **7**: 3.
Schutte, B. C., J. P. Mitros, et al. (2002). "Discovery of five conserved beta -defensin gene clusters using a computational search strategy." Proc Natl Acad Sci U S A **99**(4): 2129-33.
Seemuller, U., M. Arnhold, et al. (1986). "The acid-stable proteinase inhibitor of human mucous secretions (HUSI-I, antileukoprotease). Complete amino acid sequence as revealed by protein and cDNA sequencing and structural

homology to whey proteins and Red Sea turtle proteinase inhibitor." FEBS Lett **199**(1): 43-8.
Sehnert, B., A. Cavcic, et al. (2004). "Antileukoproteinase: modulation of neutrophil function and therapeutic effects on anti-type II collagen antibody-induced arthritis." Arthritis Rheum **50**(7): 2347-59.
Selsted, M. E., S. I. Miller, et al. (1992). "Enteric defensins: antibiotic peptide components of intestinal host defense." J Cell Biol **118**(4): 929-36.
Shai, Y. (1999). "Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides." Biochim Biophys Acta **1462**(1-2): 55-70.
Sharrocks, A. D. (2001). "The ETS-domain transcription factor family." Nat Rev Mol Cell Biol **2**(11): 827-37.
Shi, L., W. Tong, et al. (2005). "Cross-platform comparability of microarray technology: intra-platform consistency and appropriate data analysis procedures are essential." BMC Bioinformatics **6 Suppl 2**: S12.
Shimoya, K., N. Matsuzaki, et al. (1992). "Human placenta constitutively produces interleukin-8 during pregnancy and enhances its production in intrauterine infection." Biol Reprod **47**(2): 220-6.
Shine, N. R., S. C. Wang, et al. (2002). "Secretory leukocyte protease inhibitor: inhibition of human immunodeficiency virus-1 infection of monocytic THP-1 cells by a newly cloned protein." Bioorg Chem **30**(4): 249-63.
Shobokshi, A. and M. Shaarawy (2002). "Maternal serum and amniotic fluid cytokines in patients with preterm premature rupture of membranes with and without intrauterine infection." Int J Gynaecol Obstet **79**(3): 209-15.
Si-Tahar, M., D. Merlin, et al. (2000). "Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells." Gastroenterology **118**(6): 1061-71.
Simhan, H. N., M. A. Krohn, et al. (2003). "Interleukin-6 promoter -174 polymorphism and spontaneous preterm birth." Am J Obstet Gynecol **189**(4): 915-8.
Simhan, H. N., M. A. Krohn, et al. (2003). "Tumor necrosis factor-alpha promoter gene polymorphism -308 and chorioamnionitis." Obstet Gynecol **102**(1): 162-6.
Simoes, J. A., L. G. Bahamondes, et al. (2006). "A pilot clinical trial comparing an acid-buffering formulation (ACIDFORM gel) with metronidazole gel for the treatment of symptomatic bacterial vaginosis." Br J Clin Pharmacol **61**(2): 211-7.
Simon, C., A. Frances, et al. (1994). "Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function." J Clin Endocrinol Metab **78**(4): 847-54.
Simpson, A. J., G. A. Cunningham, et al. (2001). "Regulation of adenovirus-mediated elafin transgene expression by bacterial lipopolysaccharide." Hum Gene Ther **12**(11): 1395-406.
Simpson, A. J., A. I. Maxwell, et al. (1999). "Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens." FEBS Lett **452**(3): 309-13.

Simpson, A. J., W. A. Wallace, et al. (2001). "Adenoviral augmentation of elafin protects the lung against acute injury mediated by activated neutrophils and bacterial infection." J Immunol **167**(3): 1778-86.
Singh, P. K., H. P. Jia, et al. (1998). "Production of beta-defensins by human airway epithelia." Proc Natl Acad Sci U S A **95**(25): 14961-6.
Singh, P. K., B. F. Tack, et al. (2000). "Synergistic and additive killing by antimicrobial factors found in human airway surface liquid." Am J Physiol Lung Cell Mol Physiol **279**(5): L799-805.
Slater, D., W. Dennes, et al. (1999). "Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy." J Mol Endocrinol **22**(2): 125-30.
Smaill, F. (2001). "Antibiotics for asymptomatic bacteriuria in pregnancy." Cochrane Database Syst Rev(2): CD000490.
Smart, S., A. Singal, et al. (2004). "Social and sexual risk factors for bacterial vaginosis." Sex Transm Infect **80**(1): 58-62.
Soboll, G., T. M. Schaefer, et al. (2006). "Effect of toll-like receptor (TLR) agonists on TLR and microbicide expression in uterine and vaginal tissues of the mouse." Am J Reprod Immunol **55**(6): 434-46.
Sood, R., J. L. Zehnder, et al. (2006). "Gene expression patterns in human placenta." Proc Natl Acad Sci U S A **103**(14): 5478-83.
Sooranna, S. R., P. L. Grigsby, et al. (2006). "Myometrial prostaglandin E2 synthetic enzyme mRNA expression: spatial and temporal variations with pregnancy and labour." Mol Hum Reprod **12**(10): 625-31.
Spandorfer, S. D., A. Neuer, et al. (2001). "Relationship of abnormal vaginal flora, proinflammatory cytokines and idiopathic infertility in women undergoing IVF." J Reprod Med **46**(9): 806-10.
Spaziani, E. P., J. C. Tsibris, et al. (1997). "The effect of interleukin-1 beta and interleukin-4 on the expression of prostaglandin receptors EP1 and EP3 in amnion WISH cells." Am J Reprod Immunol **38**(4): 279-85.
Stallmach, T., G. Hebisch, et al. (1995). "Cytokine production and visualized effects in the feto-maternal unit. Quantitative and topographic data on cytokines during intrauterine disease." Lab Invest **73**(3): 384-92.
Steel, J. H., S. Malatos, et al. (2005). "Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor." Pediatr Res **57**(3): 404-11.
Stein, B. and A. S. Baldwin, Jr. (1993). "Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-kappa B." Mol Cell Biol **13**(11): 7191-8.
Steinborn, A., H. Gunes, et al. (1996). "Elevated placental cytokine release, a process associated with preterm labor in the absence of intrauterine infection." Obstet Gynecol **88**(4 Pt 1): 534-9.
Steinborn, A., C. von Gall, et al. (1998). "Identification of placental cytokine-producing cells in term and preterm labor." Obstet Gynecol **91**(3): 329-35.
Stephanou, A., L. Myatt, et al. (1995). "Ontogeny of the expression and regulation of interleukin-6 (IL-6) and IL-1 mRNAs by human trophoblast cells during differentiation in vitro." J Endocrinol **147**(3): 487-96.
Sternlicht, M. D. and Z. Werb (2001). "How matrix metalloproteinases regulate cell behavior." Annu Rev Cell Dev Biol **17**: 463-516.

Stjernholm-Vladic, Y., D. Stygar, et al. (2004). "Factors involved in the inflammatory events of cervical ripening in humans." Reprod Biol Endocrinol **2**: 74.

Sturm-Ramirez, K., A. Gaye-Diallo, et al. (2000). "High levels of tumor necrosis factor-alpha and interleukin-1beta in bacterial vaginosis may increase susceptibility to human immunodeficiency virus." J Infect Dis **182**(2): 467-73.

Sturm, P. D., P. Moodley, et al. (2002). "Diagnosis of bacterial vaginosis on self-collected vaginal tampon specimens." Int J STD AIDS **13**(8): 559-63.

Sun, K., R. Ma, et al. (2003). "Glucocorticoids induce cytosolic phospholipase A2 and prostaglandin H synthase type 2 but not microsomal prostaglandin E synthase (PGES) and cytosolic PGES expression in cultured primary human amnion cells." J Clin Endocrinol Metab **88**(11): 5564-71.

Sunnergren, K. P., R. A. Word, et al. (1990). "Expression and regulation of endothelin precursor mRNA in avascular human amnion." Mol Cell Endocrinol **68**(1): R7-14.

Svare, J. A., H. Schmidt, et al. (2006). "Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections." Bjog **113**(12): 1419-25.

Svinarich, D. M., R. Gomez, et al. (1997). "Detection of human defensins in the placenta." Am J Reprod Immunol **38**(4): 252-5.

Svinarich, D. M., N. A. Wolf, et al. (1997). "Detection of human defensin 5 in reproductive tissues." Am J Obstet Gynecol **176**(2): 470-5.

Tafari, N., S. M. Ross, et al. (1977). "Failure of bacterial growth inhibition by amniotic fluid." Am J Obstet Gynecol **128**(2): 187-9.

Taggart, C. C., S. A. Cryan, et al. (2005). "Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding." J Exp Med **202**(12): 1659-68.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.

Tam, M. T., M. Yungbluth, et al. (1998). "Gram stain method shows better sensitivity than clinical criteria for detection of bacterial vaginosis in surveillance of pregnant, low-income women in a clinical setting." Infect Dis Obstet Gynecol **6**(5): 204-8.

Tanaka, N., A. Fujioka, et al. (2000). "Elafin is induced in epidermis in skin disorders with dermal neutrophilic infiltration: interleukin-1 beta and tumour necrosis factor-alpha stimulate its secretion in vitro." Br J Dermatol **143**(4): 728-32.

Tang, Y. Q., J. Yuan, et al. (1999). "A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins." Science **286**(5439): 498-502.

Taniguchi, T., N. Matsuzaki, et al. (1991). "The enhanced production of placental interleukin-1 during labor and intrauterine infection." Am J Obstet Gynecol **165**(1): 131-7.

Taylor-Robinson, D., D. J. Morgan, et al. (2003). "Relation between Gram-stain and clinical criteria for diagnosing bacterial vaginosis with special reference to Gram grade II evaluation." Int J STD AIDS **14**(1): 6-10.

Terzidou, V., Y. Lee, et al. (2006). "Regulation Of The Human Oxytocin Receptor By NF-{Kappa}B And C/EBP{Beta}." J Clin Endocrinol Metab.**91**(6): 2317-26

Testi, R., D. D'Ambrosio, et al. (1994). "The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells." Immunol Today **15**(10): 479-83.

Thijssen, J. H. (2005). "Progesterone receptors in the human uterus and their possible role in parturition." J Steroid Biochem Mol Biol **97**(5): 397-400.

Thomas, G. B., A. J. Sbarra, et al. (1988). "Antimicrobial activity of amniotic fluid against Chlamydia trachomatis, Mycoplasma hominis, and Ureaplasma urealyticum." Am J Obstet Gynecol **158**(1): 16-22.

Thompson, J. E., R. J. Phillips, et al. (1995). "I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B." Cell **80**(4): 573-82.

Thompson, R. C. and K. Ohlsson (1986). "Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase." Proc Natl Acad Sci U S A **83**(18): 6692-6.

Tomee, J. F., P. S. Hiemstra, et al. (1997). "Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi." J Infect Dis **176**(3): 740-7.

Torbe, A. and R. Czajka (2004). "Proinflammatory cytokines and other indications of inflammation in cervico-vaginal secretions and preterm delivery." Int J Gynaecol Obstet **87**(2): 125-30.

Totzke, G., F. Essmann, et al. (2006). "A Novel Member of the I{kappa}B Family, Human I{kappa}B-{zeta}, Inhibits Transactivation of p65 and Its DNA Binding." J Biol Chem **281**(18): 12645-54.

Trautman, M. S., D. J. Dudley, et al. (1992). "Amnion cell biosynthesis of interleukin-8: regulation by inflammatory cytokines." J Cell Physiol **153**(1): 38-43.

Trautman, M. S., S. S. Edwin, et al. (1996). "Prostaglandin H synthase-2 in human gestational tissues: regulation in amnion." Placenta **17**(4): 239-45.

Tremblay, G. M., E. Vachon, et al. (2002). "Inhibition of human neutrophil elastase-induced acute lung injury in hamsters by recombinant human pre-elafin (trappin-2)." Chest **121**(2): 582-8.

Tromp, G., H. Kuivaniemi, et al. (2004). "Genome-wide expression profiling of fetal membranes reveals a deficient expression of proteinase inhibitor 3 in premature rupture of membranes." Am J Obstet Gynecol **191**(4): 1331-8.

Tsutsumi-Ishii, Y. and I. Nagaoka (2002). "NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation." J Leukoc Biol **71**(1): 154-62.

Tsutsumi-Ishii, Y. and I. Nagaoka (2003). "Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production." J Immunol **170**(8): 4226-36.

Ulug, U., S. Goldman, et al. (2001). "Matrix metalloproteinase (MMP)-2 and MMP-9 and their inhibitor, TIMP-1, in human term decidua and fetal membranes: the effect of prostaglandin F(2alpha) and indomethacin." Mol Hum Reprod **7**(12): 1187-93.

Underwood, M. A., W. M. Gilbert, et al. (2005). "Amniotic fluid: not just fetal urine anymore." J Perinatol **25**(5): 341-8.

Vachon, E., Y. Bourbonnais, et al. (2002). "Anti-inflammatory effect of pre-elafin in lipopolysaccharide-induced acute lung inflammation." Biol Chem **383**(7-8): 1249-56.
Valore, E. V., C. H. Park, et al. (2002). "Antimicrobial components of vaginal fluid." Am J Obstet Gynecol **187**(3): 561-8.
Valore, E. V., C. H. Park, et al. (1998). "Human beta-defensin-1: an antimicrobial peptide of urogenital tissues." J Clin Invest **101**(8): 1633-42.
Valore, E. V., D. J. Wiley, et al. (2006). "Reversible deficiency of antimicrobial polypeptides in bacterial vaginosis." Infect Immun **74**(10): 5693-702.
van Bergen, B. H., M. P. Andriessen, et al. (1996). "Expression of SKALP/elafin during wound healing in human skin." Arch Dermatol Res **288**(8): 458-62.
Vankeerberghen, A., H. Nuytten, et al. (2005). "Differential induction of human beta-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells." J Periodontol **76**(8): 1293-303.
Vince, G., S. Shorter, et al. (1992). "Localization of tumour necrosis factor production in cells at the materno/fetal interface in human pregnancy." Clin Exp Immunol **88**(1): 174-80.
Vives, A., J. Balasch, et al. (1999). "Type-1 and type-2 cytokines in human decidual tissue and trophoblasts from normal and abnormal pregnancies detected by reverse transcriptase polymerase chain reaction (RT-PCR)." Am J Reprod Immunol **42**(6): 361-8.
Vogelmeier, C., R. Buhl, et al. (1990). "Aerosolization of recombinant SLPI to augment antineutrophil elastase protection of pulmonary epithelium." J Appl Physiol **69**(5): 1843-8.
Vogelmeier, C., A. Gillissen, et al. (1996). "Use of secretory leukoprotease inhibitor to augment lung antineutrophil elastase activity." Chest **110**(6 Suppl): 261S-266S.
Wahl, C., S. Liptay, et al. (1998). "Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B." J Clin Invest **101**(5): 1163-74.
Wang, X., X. Li, et al. (2003). "Up-regulation of secretory leukocyte protease inhibitor (SLPI) in the brain after ischemic stroke: adenoviral expression of SLPI protects brain from ischemic injury." Mol Pharmacol **64**(4): 833-40.
Watts, D. H., M. A. Krohn, et al. (1992). "The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor." Obstet Gynecol **79**(3): 351-7.
Weber, C. K., S. Liptay, et al. (2000). "Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta." Gastroenterology **119**(5): 1209-18.
Wehkamp, J., K. Fellermann, et al. (2005). "Human defensins in Crohn's disease." Chem Immunol Allergy **86**: 42-54.
Weiyuan, Z. and W. Li (1998). "Study of interleukin-6 and tumor necrosis factor-alpha levels in maternal serum and amniotic fluid of patients with premature rupture of membranes." J Perinat Med **26**(6): 491-4.
Wennerholm, U. B., B. Holm, et al. (1998). "Interleukin-1alpha, interleukin-6 and interleukin-8 in cervico/vaginal secretion for screening of preterm birth in twin gestation." Acta Obstet Gynecol Scand **77**(5): 508-14.

Wenstrom, K. D., W. W. Andrews, et al. (1996). "Elevated amniotic fluid interleukin-6 levels at genetic amniocentesis predict subsequent pregnancy loss." Am J Obstet Gynecol **175**(4 Pt 1): 830-3.
Westin, U., A. Polling, et al. (1999). "Identification of SLPI (secretory leukocyte protease inhibitor) in human mast cells using immunohistochemistry and in situ hybridisation." Biol Chem **380**(4): 489-93.
Whittle, W. L., W. Gibb, et al. (2000). "The characterization of human amnion epithelial and mesenchymal cells: the cellular expression, activity and glucocorticoid regulation of prostaglandin output." Placenta **21**(4): 394-401.
Whitworth, M. K., I. Pafilis, et al. (2007). "Cervical leukocyte sub-populations in idiopathic preterm labour." J Reprod Immunol.
Wiedow, O., J. Harder, et al. (1998). "Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes." Biochem Biophys Res Commun **248**(3): 904-9.
Wiedow, O., J. M. Schroder, et al. (1990). "Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence." J Biol Chem **265**(25): 14791-5.
Wilkinson, D., N. Ndovela, et al. (1997). "Tampon sampling for diagnosis of bacterial vaginosis: a potentially useful way to detect genital infections?" J Clin Microbiol **35**(9): 2408-9.
Williams, S. E., T. I. Brown, et al. (2006). "SLPI and elafin: one glove, many fingers." Clin Sci (Lond) **110**(1): 21-35.
Wingens, M., B. H. van Bergen, et al. (1998). "Induction of SLPI (ALP/HUSI-I) in epidermal keratinocytes." J Invest Dermatol **111**(6): 996-1002.
Winkler, M., D. C. Fischer, et al. (1998). "Interleukin-1beta and interleukin-8 concentrations in the lower uterine segment during parturition at term." Obstet Gynecol **91**(6): 945-9.
Witthoft, T., C. S. Pilz, et al. (2005). "Enhanced human beta-defensin-2 (hBD-2) expression by corticosteroids is independent of NF-kappaB in colonic epithelial cells (CaCo2)." Dig Dis Sci **50**(7): 1252-9.
Word, R. A., K. E. Kamm, et al. (1990). "Endothelin increases cytoplasmic calcium and myosin phosphorylation in human myometrium." Am J Obstet Gynecol **162**(4): 1103-8.
Yamaguchi, Y., T. Nagase, et al. (2002). "Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice." J Immunol **169**(5): 2516-23.
Yamamoto, M., S. Yamazaki, et al. (2004). "Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta." Nature **430**(6996): 218-22.
Yamazaki, S., T. Muta, et al. (2005). "Stimulus-specific induction of a novel nuclear factor-kappaB regulator, IkappaB-zeta, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization." J Biol Chem **280**(2): 1678-87.
Yamazaki, S., T. Muta, et al. (2001). "A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei." J Biol Chem **276**(29): 27657-62.
Yang, D., A. Biragyn, et al. (2004). "Multiple Roles of Antimicrobial Defensins, Cathelicidins, and Eosinophil-Derived Neurotoxin in Host Defense*." Annu Rev Immunol **22**: 181-215.

Yang, D., O. Chertov, et al. (1999). "Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6." Science **286**(5439): 525-8.
Yang, L., T. M. Weiss, et al. (2000). "Crystallization of antimicrobial pores in membranes: magainin and protegrin." Biophys J **79**(4): 2002-9.
Yang, Y., K. K. Yelavarthi, et al. (1993). "Molecular, biochemical, and functional characteristics of tumor necrosis factor-alpha produced by human placental cytotrophoblastic cells." J Immunol **150**(12): 5614-24.
Yao, Z., S. L. Painter, et al. (1995). "Human IL-17: a novel cytokine derived from T cells." J Immunol **155**(12): 5483-6.
Ye, P., P. B. Garvey, et al. (2001). "Interleukin-17 and lung host defense against Klebsiella pneumoniae infection." Am J Respir Cell Mol Biol **25**(3): 335-40.
Yellon, S. M., A. M. Mackler, et al. (2003). "The role of leukocyte traffic and activation in parturition." J Soc Gynecol Investig **10**(6): 323-38.
Yoon, B. H., S. Y. Oh, et al. (2001). "An elevated amniotic fluid matrix metalloproteinase-8 level at the time of mid-trimester genetic amniocentesis is a risk factor for spontaneous preterm delivery." Am J Obstet Gynecol **185**(5): 1162-7.
Yoon, B. H., R. Romero, et al. (2003). "The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor." Am J Obstet Gynecol **189**(4): 919-24.
Yordy, J. S. and R. C. Muise-Helmericks (2000). "Signal transduction and the Ets family of transcription factors." Oncogene **19**(55): 6503-13.
Young, A., A. J. Thomson, et al. (2002). "Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term." Biol Reprod **66**(2): 445-9.
Zaidi, S. H., X. M. You, et al. (2000). "Suppressed smooth muscle proliferation and inflammatory cell invasion after arterial injury in elafin-overexpressing mice." J Clin Invest **105**(12): 1687-95.
Zasloff, M. (2002). "Antimicrobial peptides in health and disease." N Engl J Med **347**(15): 1199-200.
Zhang, M., Z. Zou, et al. (1995). "Differential expression of elafin in human normal mammary epithelial cells and carcinomas is regulated at the transcriptional level." Cancer Res **55**(12): 2537-41.
Zhang, Q., K. Shimoya, et al. (2001). "Production of secretory leukocyte protease inhibitor by human amniotic membranes and regulation of its concentration in amniotic fluid." Mol Hum Reprod **7**(6): 573-9.
Zhang, Y., D. L. DeWitt, et al. (1997). "Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinases." J Clin Invest **99**(5): 894-900.
Zhang, Z., A. Andoh, et al. (2005). "Interleukin-17 and lipopolysaccharides synergistically induce cyclooxygenase-2 expression in human intestinal myofibroblasts." J Gastroenterol Hepatol **20**(4): 619-27.
Zhao, C., I. Wang, et al. (1996). "Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells." FEBS Lett **396**(2-3): 319-22.
Zimmermann, G. R., P. Legault, et al. (1995). "Solution structure of bovine neutrophil beta-defensin-12: the peptide fold of the beta-defensins is identical to that of the classical defensins." Biochemistry **34**(41): 13663-71.

Bibliography

Benirschke, K and Kaufmann, Pathology of the Human Placenta, Springer Verlag, 1995.

Appendix 1: Materials

TISSUE COLLECTION	SOURCE
Phosphate Buffered Saline	Sigma-Aldrich, Poole, Dorset, UK
RNAlater	Ambion, Austin, Texas, USA
Neutral Buffered Formalin	See Appendix 2
Tri-reagent	Sigma-Aldrich, Poole, Dorset, UK
Ethanol	Hayman Ltd., Essex, UK
Tampons (Tampax Regular)	Proctor and Gamble, Surrey, UK
Glass microscope slides	Fischer Scientific, Loughborough, UK
Acetic Acid	BDH Laboratory Supplies, Poole, UK
Gram Stain Kit	BIOS Europe, Skelmersdale, Lancs,UK
Pertex mounting medium	Cellpath plc, Hemel Hempsted, UK
Cover slips	Fischer Scientific, Loughborough, UK

TISSUE & CELL CULTURE	SOURCE
FL cells	LGC Promochem, Teddington, UK
WISH cells	LGC Promochem, Teddington, UK
He-La cells	LGC Promochem, Teddington, UK
VK2 E6/E7	LGC Promochem, Teddington, UK
ECT E6/E7	LGC Promochem, Teddington, UK
END E6/E7	LGC Promochem, Teddington, UK

TISSUE & CELL CULTURE	SOURCE
Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Poole, Dorset, UK
EDTA	Sigma-Aldrich, Poole, Dorset, UK
Dispase	Gibco, Paisley, UK
RPMI 1640 medium	Sigma-Aldrich, Poole, Dorset, UK
Fetal Calf Serum	Mycoplex, Teddington, UK
Penicillin	Sigma-Aldrich, Poole, Dorset, UK
Streptomycin	Sigma-Aldrich, Poole, Dorset, UK
Gentamycin	Sigma-Aldrich, Poole, Dorset, UK
L-Glutamine	Sigma-Aldrich, Poole, Dorset, UK
Trypan Blue	Sigma-Aldrich, Poole, Dorset, UK
Culture plates (6 well and 12 well)	Corning Costar, High Wycombe, UK
Keratinocyte Serum Free Medium	Invitrogen, Paisley, Scotland, UK
Bovine Pituitary Extract	Invitrogen, Paisley, Scotland, UK
Epidermal Growth Factor	Invitrogen, Paisley, Scotland, UK
RNAlater	Ambion, Austin, Texas, USA
Protein Lysis Buffer	See Appendix 2
Minitab protease inhibitor tablets	Roche Biochemicals, Burgess Hill, UK
Interleukin-1β (IL-1β)	Peprotech EC, London, UK

TISSUE & CELL CULTURE	SOURCE
Interleukin-17 (IL-17)	Peprotech EC, London, UK
Calcium Chloride	Sigma-Aldrich, Poole, Dorset, UK
Tumour Necrosis Factor α (TNFα)	Peprotech EC, London, UK
Interleukin-8 (CXCL8)	Peprotech EC, London, UK
Bone Morphogenic Protein 2 (BMP2)	Peprotech EC, London, UK
HBD2	Peprotech EC, London, UK
SLPI	HycultBiotechnology, Uden, Netherlands
Lipopolysaccharide (LPS) – E. coli	Sigma-Aldrich, Poole, Dorset, UK
Lipoteichoic acid (LTA)	Sigma-Aldrich, Poole, Dorset, UK
Progesterone	Sigma-Aldrich, Poole, Dorset, UK
Indomethacin	Sigma-Aldrich, Poole, Dorset, UK
Dexamethasone	Sigma-Aldrich, Poole, Dorset, UK
Sulfasalazine	Sigma-Aldrich, Poole, Dorset, UK
NFKB Activation Inhibitor	Calbiochem, Darmstadt, Germany
BAY 11-7082	Calbiochem, Darmstadt, Germany
SB2030580	Calbiochem, Darmstadt, Germany
PD8059	Calbiochem, Darmstadt, Germany
JNK II Inhibitor	Calbiochem, Darmstadt, Germany

RNA EXTRACTION AND PCR	SOURCE
RNeasy mini RNA extraction kit	Qiagen, Crawley, West Sussex, UK
TRI reagent	Ambion, Austin, Texas, USA
Chloroform	BDH Laboratory Supplies, Poole, UK
DNase I	Qiagen, Crawley, West Sussex, UK
Ethanol	Hayman Ltd., Essex, UK
RNA_{6000}nano chips and reagents	Agilent Biotechnologies, Stockport, Cheshire,UK
Mineral Oil	Sigma-Aldrich, Poole, Dorset, UK
Stratgene PCR Mastermix	Applied Biosystems, Warrington, Cheshire, UK
18s Control Primer and Probe	Applied Biosystems, Warrington, Cheshire, UK
CD69, BMP2, NFKBIZ and latterly used HBD2 and HBD3 Primers and Probes	MWG-Biotech, Ebersberg, Germany
All other Primers and Probes	Biosource, Nivelles, Belgium

ELISA	SOURCE
96 well assay plates	Nunc Maxi-Sorp, Gibco, Paisley, UK
HBD2 Assay Development kit	Peprotech EC, London, UK
HBD3 Assay Development kit	Peprotech EC, London, UK
IL-8 Capture Antibody	R&D Systems, Oxford, UK
IL-8 Standard	Toray Industries, Tokyo, Japan
IL-8 Detection antibody	R&D Systems, Oxford, UK

ELISA	SOURCE
IL-1β Capture Antibody	R&D Systems, Oxford, UK
IL-1β Standard	R&D Systems, Oxford, UK
IL-1β Detection Antibody	R&D Systems, Oxford, UK
IL-1RA Capture Antibody	R&D Systems, Oxford, UK
IL-1RA Standard	R&D Systems, Oxford, UK
IL-1RA Detection Antibody	R&D Systems, Oxford, UK
TNFα Capture Antibody	R&D Systems, Oxford, UK
TNFα Standard	R&D Systems, Oxford, UK
TNFα Detection Antibody	R&D Systems, Oxford, UK
SLPI ELISA	HycultBiotechnology, Uden, Netherlands
Elafin ELISA	HycultBiotechnology, Uden, Netherlands
Phosphate Buffered Saline tablets	Sigma-Aldrich, Poole, Dorset, UK
Tween	Sigma-Aldrich, Poole, Dorset, UK
ELISA Buffer	See Appendix 2
Dry coat	See Appendix 2
Wash Buffer	See Appendix 2

Substrate	See Appendix 2
2N sulphuric acid	BDH Laboratory Supplies, Poole, UK
Bovine Serum Albumin	Sigma-Aldrich, Poole, Dorset, UK
Reagent A and S	Bio-Rad Laboratories, Hemel Hempstead, UK
Reagent B	Bio-Rad Laboratories, Hemel Hempstead, UK

IMMUNOHISTOCHEMISTRY	**SOURCE**
Xylene	BDH Laboratory Supplies, Poole, UK
Ethanol	Hayman Ltd., Essex, UK
Harris's Haematoxylin	Pioneer Research Chemicals Ltd, Colchester, UK
Acid Alcohol	BDH Laboratory Supplies, Poole, UK
Scott's tap water	See Appendix 2
Eosin	Pioneer Research Chemicals Ltd, Colchester, UK
Pertex	Cellpath plc, Hemel Hempsted, UK
Neutral Buffered Formalin	See Appendix 2

IMMUNOHISTOCHEMISTRY	SOURCE
Triton	Sigma-Aldrich, Poole, Dorset, UK
Phosphate Buffered Saline	See Appendix 2
Phosphate Buffered Saline + Tween	See Appendix 2
0.01M sodium citrate	See Appendix 2
Hydrogen Peroxide	BDH Laboratory Supplies, Poole, UK
Methanol	BDH Laboratory Supplies, Poole, UK
Blocking serum	See Appendix 2
Anti-Cytokeratin	Dako Ltd., Cambridge, UK
Rabbit anti-mouse Antibody	Dako Ltd., Cambridge, UK
Avidin-Biotin complex (ABC)	Dako Ltd., Cambridge, UK.
Diaminobenzidine	Dako Ltd., Cambridge, UK

MICROARRAY	SOURCE
Low RNA Input Fluorescent Amplification kit	Agilent Biotechnologies, Stockport, Cheshire,UK
In-Situ Hybridisation Kit plus	Agilent Biotechnologies, Stockport, Cheshire,UK

MICROARRAY	SOURCE
Cyanine 3-CTP (10.0mM)	PerkinElmer/NEN Life Sciences
Cyanine 5-CTP(10.0mM)	PerkinElmer/NEN Life Sciences
Ethanol	BDH Laboratory Supplies, Poole, UK
Qiagen Rneasy mini kit	Qiagen, Crawley, West Sussex, UK
20x Saline-Sodium-Phosphate-EDTA Buffer	Amresco, Solon, Ohio, USA
20% N-lauroylsarcosine	Sigma-Aldrich, Poole, Dorset, UK
Agilent Stabilization and Drying Solution	Agilent Biotechnologies, Stockport, Cheshire,UK

Appendix 2: Recipes for Solutions

All dilutions are in deionized water, unless otherwise stated.

Neutral buffered formalin (NBF)

In 1 litre:

6.5g Na_2HPO_4 (BDH Laboratory Supplies, Poole, UK)

4.5g $NaH_2PO_4.2H_20$ (BDH Laboratory Supplies, Poole, UK)

100ml 40% formaldehyde (BDH Laboratory Supplies, Poole, UK)

900ml distilled water

Protein Lysis Buffer

150 mmol/l NaCl

50 mmol/l Tris pH 7.4

10 mmol/l EDTA

10 mmol/l EGTA

0.6% Nonidet P40 (Roche Biochemicals, Burgess Hill, UK)

10% Glycerol (Sigma-Aldrich, Poole, Dorset, UK)

10µg Peptain (Sigma-Aldrich, Poole, Dorset, UK)

1mM PMSF (Sigma-Aldrich, Poole, Dorset, UK)

1 Protease Inhibitor Mini Tablet per 10ml lysis buffer (Roche Biochemicals, Burgess Hill, UK)

ELISA buffer

In 1 litre:

12.1g Tris (Sigma-Aldrich, Poole, Dorset, UK)

2g BSA (Sigma-Aldrich, Poole, Dorset, UK)

9g NaCl (Sigma-Aldrich, Poole, Dorset, UK)

0.7g EDTA (Sigma-Aldrich, Poole, Dorset, UK)

300µl phenol red (Flow Laboratories, UK)

1ml preservatives (Boehringer, Mannheim, Germany)

pH 7.2

Dry Coat

In 1 litre:

20g polyvinylpyrollidone 2% (Sigma-Aldrich, Poole, Dorset, UK)

5g BSA (Sigma-Aldrich, Poole, Dorset, UK)

1ml preservatives (Boehringer, Mannheim, Germany)

1.9g EDTA (Sigma-Aldrich, Poole, Dorset, UK)

6.1g Tris (Sigma-Aldrich, Poole, Dorset, UK)

ELISA wash buffer

In 1 litre:

5ml Tween 20 (Sigma-Aldrich, Poole, Dorset, UK)

90g NaCl (Sigma-Aldrich, Poole, Dorset, UK)

12.1g Tris (Sigma-Aldrich, Poole, Dorset, UK)

pH 7-7.5

Dilute 1:20 to use

ELISA substrate

1ml tetramethyl benzidine: 1ml urea hydrogen peroxide : 10ml sodium acetate

100mM sodium acetate

In 1 litre:

13.6g sodium trihydrate (Sigma-Aldrich, Poole, Dorset, UK)

1ml preservatives (Boehringer, Manheim, Germany)

pH 6

Tetramethyl Benzidine

3mg/ml in dimethylformamide (Sigma-Aldrich, Poole, Dorset, UK)

Urea Hydrogen Peroxide

0.5% in 50mM sodium acetate (Sigma-Aldrich, Poole, Dorset, UK)

pH6

Phosphate buffered saline

In 1 litre:

5 PBS tablets (Sigma-Aldrich, Poole, Dorset, UK)

pH 7.4-7.6

Phosphate buffered saline + Tween

In 1 litre:

5 PBS tablets (Sigma-Aldrich, Poole, Dorset, UK)

100µl Tween-20 (Sigma-Aldrich, Poole, Dorset, UK)

pH 7.4-7.6

Scott's Tap Water

In 1litre:

3.2g Sodium Bicarbonate (Sigma-Aldrich, Poole, Dorset, UK)

36.1g Magnesium Sulphate (Sigma-Aldrich, Poole, Dorset, UK)

0.1M sodium citrate buffer

In 1litre:

29.4g Tri-sodium citrate (BDH Laboratory Supplies, Poole, UK)

0.1g sodium azide (Sigma-Aldrich, Poole, Dorset, UK)

pH 6

Dilute 1:10 to use

Blocking Serum

20ml Normal Rabbit Serum (Vector Laboratories, Peterborough, UK)

80 ml Phosphate Buffered Saline (Sigma-Aldrich, Poole, Dorset, UK)

5g Bovine Serum Albumin (Sigma-Aldrich, Poole, Dorset, UK)

Appendix 3: Patient Information Leaflets and Consent Forms

Patient Information Leaflet

Study 1: Natural Antimicrobial Proteins in Labour

30/06/04

Natural Antimicrobial Proteins in Pregnancy

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it will involve. Please read this information carefully and ask us if anything is not clear.

What is the purpose of the study?

At the Simpson Centre for Reproductive Health we are researching premature birth. Studies have shown that the cause for this is sometimes infection. We think that both the mother and the baby produce substances that act as natural antibiotics to help protect the pregnancy, and when these defences are overcome, infection can take hold and may cause labour to start. Exactly how these natural antibiotics work is unknown, but we think they may have an important role in maintaining pregnancy. We are currently performing studies investigating this.

How are we doing the research?

Many different tissues produce these natural antibiotics, called natural antimicrobial proteins. We would like to collect some samples from you to look at them. We will then study these to see if natural antimicrobial proteins are present and how they work.

Why is it important?

Premature birth occurs in about 10% of pregnancies. Despite advances in the care of very small or premature babies, it still is the leading cause of babies dying in this country. Although we know some of the risk factors for developing preterm labour, exactly why it happens is still a mystery, and it remains difficult to predict and to treat.

What does it involve for me?

We are collecting samples from women who are delivered by caesarean section. We would like to collect a small amount of the "waters" or amniotic fluid that surrounds your baby. This is done by sucking up a little of the fluid with a syringe, just before your baby is delivered. After delivery we are taking samples of the after-birth (the placenta) and membranes that surrounded your baby. We also want to take a small sample of the umbilical cord that attached your baby to the placenta. None of this involves any additional procedures or alters delivery or care of your baby in any way.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in this pregnancy, or any future pregnancies.

What will happen to the results of this research study?

This research study is taking place over a three-year period. Any results will be published in peer reviewed medical and scientific journals. Updates to the progress of this, and other studies from this department can be found on our website at www.piggybankkids.org

Who is organizing and funding the research?

This research study is funded by The Jennifer Brown Research Trust, which is supported by the PiggyBankKids appeal (registered charity number 1092312). The aim of the charity is to find solutions to pregnancy difficulties and save newborn lives. The Local Ethics Committee has approved this study.

Will my taking part in this study be kept confidential?

If you consent to take part in the research, we will send a letter to your GP to let him/her know of your involvement. Your medical records may be inspected by the researchers, where it is relevant to analyzing the results. All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Who can I contact for further information?

If you would like to ask any questions about the study please contact either Dr Sarah Stock on 0131 242 6425 or Dr Anne Armstrong on 0131 242 6424 or via hospital switchboard. If you would like to speak to someone not in any way involved in the study please contact Dr Christine West on telephone 0131 242 2525.

What will happen to the samples that I give?

After the study is complete any remaining fresh or frozen samples will be destroyed. However some small tissue samples will have been made in to slides and some samples will have protein extracts taken from them. We ask if we can keep these samples to use in future similar research studies. The exact nature of these studies will depend upon future scientific advances, and some may be carried out by other researchers.

The Jennifer Brown Research Trust

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We're on the Web!

See us at:

www.piggybankkids.org

Consent Form

Study Title: Natural Antimicrobial Proteins in Pregnancy
Name of Researcher: Dr Sarah Stock

1. I confirm I have read and understand the information sheet dated 30/06/04 (Natural Antimicrobial Proteins In Labour) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that my medical notes may be looked at by the researchers involved in the study or by the regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to have access to my records.

4. I agree to a letter being sent to my General Practitioner to notify them about my participation in this study.

5. I understand that fresh and frozen tissue samples will be destroyed at the end of the study. However I agree that slides and certain protein extracts, made from the tissue that I have given, may be kept by Professor Andrew Calder at the Centre for Reproductive Biology, University of Edinburgh, for possible use in future similar projects. I understand that some of these projects may be carried out by researchers other than Professor Calder's laboratory who ran the first project.

6. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

7. I agree to take part in this study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person Taking Consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Samples: Amniotic Fluid **Fixed specimen placenta/membranes**

Fresh specimen placenta membranes (*circle as appropriate*)

Patient Information Leaflet

Study 2: Natural Antimicrobial Proteins in Vaginal Secretions

10th January 2006

Natural Antimicrobial Proteins in Pregnancy

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it will involve. Please read this information carefully and ask us if anything is not clear.

What is the purpose of the study?

At the Simpson Centre for Reproductive Health we are researching premature birth. Studies have shown that the cause for this is sometimes infection. We think that both the mother and the baby produce substances that act as natural antibiotics to help protect the pregnancy, and when these defences are overcome, infection can take hold and may cause labour to start. Exactly how these natural antibiotics work is unknown, but we think they may have an important role in maintaining pregnancy. We are currently performing studies investigating this.

How are we doing the research?

Many different tissues produce these natural antibiotics, called natural antimicrobial proteins. We think that they are present in the vagina, and may help stop infections from the vagina, traveling up to affect your baby. We would like to collect some samples of vaginal fluid from you. We will then study the amounts of natural antimicrobial proteins that are present, how they work, and what may control their production. We will also investigate whether these proteins can be used as a marker to predict premature labour.

Why is it important?

Premature birth occurs in about 10% of pregnancies. Despite advances in the care of very small or premature babies, it still is the leading cause of babies dying in this country. Although we know some of the risk factors for developing preterm labour, exactly why it happens is still a mystery, and it remains difficult to predict and to treat.

What does it involve for me?

We are asking women who are booked for delivery in Edinburgh to take part. Firstly, we would like a sample of vaginal fluid. This is collected yourself, by simply inserting a tampon that we provide into the vagina. You can remove it about fifteen minutes later, into a specimen pot provided. This will not harm the pregnancy in any way, and should not be painful or uncomfortable.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in this pregnancy, or any future pregnancies.

What will happen to the results of this research study?

This research study is taking place over a three-year period. Any results will be published in peer reviewed medical and scientific journals. Updates to the progress of this, and other studies from this department can be found on our website at www.piggybankkids.org

Will my taking part in this study be kept confidential?

If you consent to take part in the research, we will send a letter to your GP to let him/her know of your involvement. Your medical records may be inspected by the researchers, where it is relevant to analyzing the results. All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

What will happen to the samples that I give?

After the study is we ask if we can keep any remaining vaginal fluid samples to use in future similar research studies. The exact nature of these studies will depend upon future scientific advances, and some may be carried out by other researchers.

Who is organizing and funding the research?

This research study is funded by The Jennifer Brown Research Trust, which is supported by the PiggyBankKids appeal (registered charity number 1092312). The aim of the charity is to find solutions to pregnancy difficulties and save newborn lives. The Local Ethics Committee has approved this study.

Who can I contact for further information?

If you would like to ask any questions about the study please contact either Dr Sarah Stock on 0131 242 6425 or Dr Anne Armstrong on 0131 242 6424 or via hospital switchboard. If you would like to speak to someone not in any way involved in the study please contact Dr Christine West on telephone 0131 242 2525.

The Jennifer Brown Research Trust

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Phone:
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E-Mail: sarah.stock@ed.ac.uk

We're on the Web!

See us at:

www.piggybankkids.org

Consent Form

Study Title: Natural Antimicrobial Proteins in Pregnancy
Name of Researcher: Dr Sarah Stock

1. I confirm I have read and understand the information sheet dated 09/09/04 (Natural Antimicrobial Proteins Throughout Pregnancy) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that my medical notes may be looked at by the researchers involved in the study or by the regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to have access to my records.

4. I agree to a letter being sent to my General Practitioner to notify them about my participation in this study.

5. I understand that fresh and frozen tissue samples will be destroyed at the end of the study. However I agree that slides and certain protein extracts, made from the tissue that I have given, may be kept by Professor Andrew Calder at the Centre for Reproductive Biology, University of Edinburgh, for possible use in future similar projects. I understand that some of these projects may be carried out by researchers other than Professor Calder's laboratory who ran the first project.

6. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

7. I agree to take part in this study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person Taking Consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix 4: Microarray Quality Control Figures

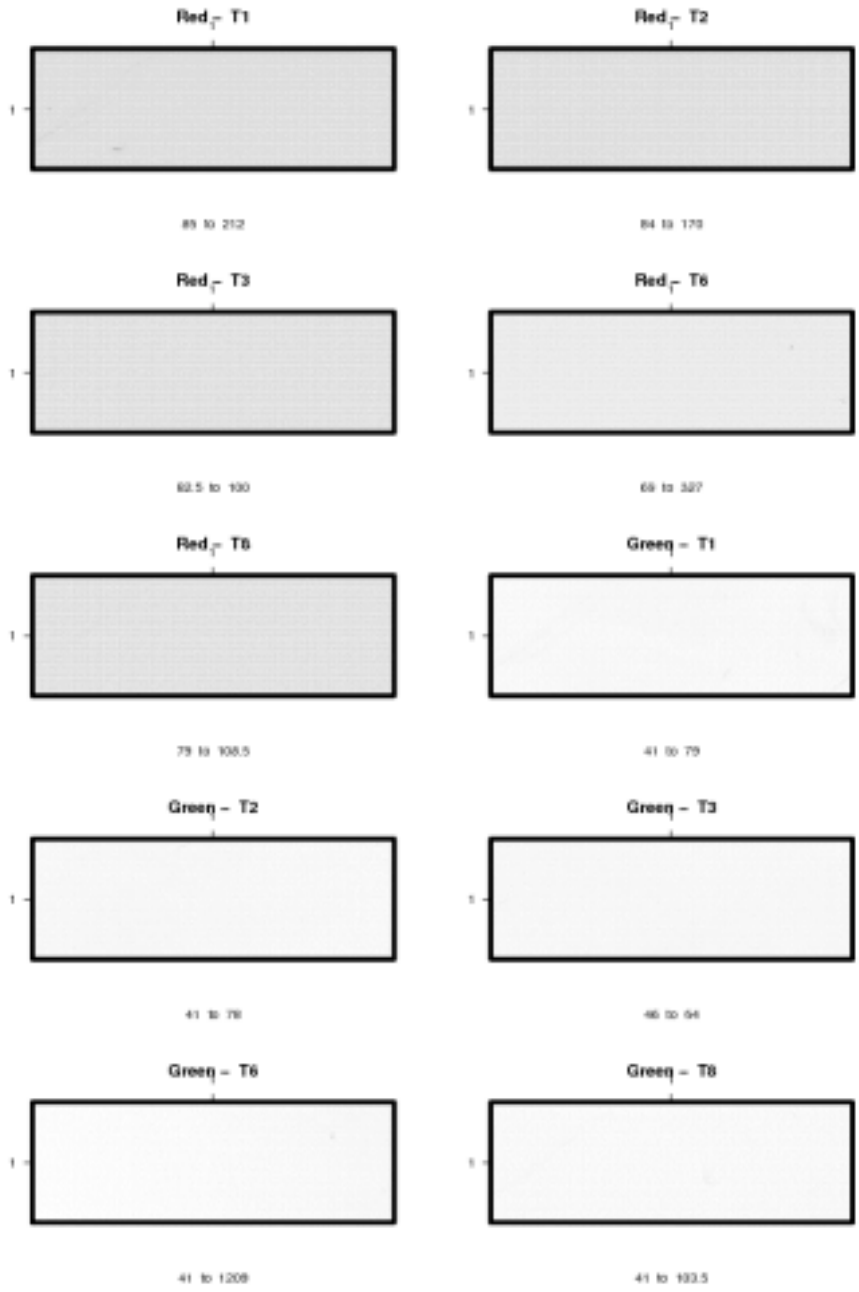

Figure A4.1

Plots of background signal intensities by position on microarray. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

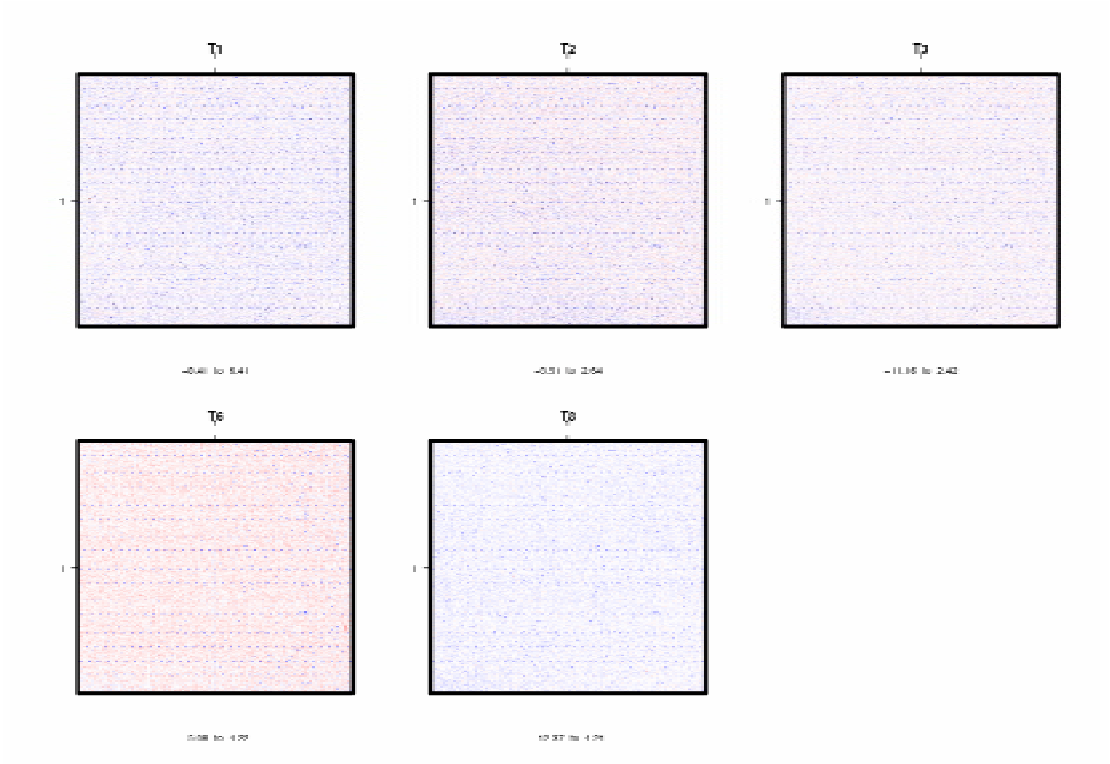

Figure A4.2

Plots of expression log2 ratios (pre-normalisation) by position on microarray. T1= 1 hour timepoint; T2= 2 hour timepoint; T3=3 hour timepoint; T6= 6 hour timepoint and T8=8 hour timepoint.

Appendix 5: Publications

BASIC SCIENCE: OBSTETRICS

Natural antimicrobial production by the amnion

Sarah J. Stock, MBChB; Rodney W. Kelly, PhD; Simon C. Riley, PhD; Andrew A. Calder, MD

OBJECTIVE: The purpose of this study was to determine the expression of natural antimicrobials in primary cultured amnion epithelial cells and to examine their regulation by interleukin-1 beta (IL-1β).

STUDY DESIGN: Primary amnion epithelial cells were cultured from samples that were obtained at prelabor cesarean section (n = 12) and stimulated with IL-1β. Natural antimicrobial messenger RNA expression was determined by real-time quantitative polymerase chain reaction, and protein was measured by enzyme-linked immunosorbent assay. Data was analyzed by analysis of variance.

RESULTS: Primary amnion epithelial cells express messenger RNA for human beta defensin (HBD) 1 to 3, secretory leukocyte protease inhibitor and elafin, but not HBD4. IL-1β 10 ng/mL stimulates HBD2 messenger RNA in a biphasic pattern, with a 51-fold increase at 6 hours and a 67-fold at 12 hours ($P < .001$). HBD2 protein production is significantly increased by 24 hours ($P < .05$).

CONCLUSION: The amnion produces potent natural antimicrobials that may help protect the pregnancy from infection. HBD2 production is dramatically upregulated by the labor-associated inflammatory cytokine IL-1β.

Key words: amnion, defensin, infection, natural antimicrobial, pregnancy

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Labor is an inflammatory process that is characterized by leukocyte invasion of the uterine tissue, and increased cytokine and prostaglandin production.[1] Intrauterine infection in pregnancy can stimulate this process prematurely and is responsible for approximately one third of all cases of preterm labor.[2] Prevention of ascending infections is paramount in maintaining a healthy pregnancy.

Natural antimicrobials are peptides that are essential components of the innate immune system that provide broad-spectrum protection against bacteria, yeasts, and some viruses.[3] The human beta defensins (HBDs) are a major family of vertebrate natural antimicrobials; HBD1-4 are expressed widely at mucosal surfaces.[4] In addition to their antimicrobial activity, HBDs have chemoattractant properties that suggest that they interact between the innate and adaptive immune systems.[5,6] A related family of molecules with important antimicrobial activities is the antileukoproteinases.[7] This group includes elafin (skin-derived antiproteinase) and secretory leukocyte protease inhibitor (SLPI).

Natural antimicrobial proteins are expressed throughout the nonpregnant female reproductive tract.[8,9] In pregnancy, natural antimicrobials are found in the amniotic fluid[10-12] and have been localized in the placenta, decidua, and fetal membranes.[13-15]

The amnion is positioned critically between the normally sterile amniotic cavity and the contaminated extrauterine environment, but amniotic production of natural antimicrobials has not been elucidated. The bacterial product lipopolysaccharide has been shown to stimulate HBD3 messenger RNA (mRNA) in so-called amnion-derived FL cells.[17] However, this cell line is derived from cervical He-La cell contaminants[19] (www.atcc.org), thus the validity of this finding is unclear. The current study examined natural antimicrobial expression and regulation in primary cultured amnion cells. We also compared the expression with that in the contaminated FL cell lines and the similarly contaminated WISH cell line[20] (www.atcc.org) to determine whether they are a suitable model for the study of amniotic innate immune responses.

Methods

Samples

The Lothian Local Research Ethics Committee approved this study, and written informed consent was obtained from all participants. Fetal membranes were collected from 12 women who underwent elective cesarean section at 39 weeks of gestation. All of the women had uncomplicated singleton pregnancies, with no signs of labor or infection. Amnion for culture was stripped from underlying chorion, washed, and transported to the

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TABLE 1
Sequences of primers and probes for Taqman polymerase chain reaction

Variable	Forward primer	Reverse primer	Probe
HBD1	TCAGCAGTGGAGGCAATG	CCTCTGTAACAGGTGCCTTGAAT	TCTATTCTGCCTGCCGAATCTTTACCAA
HBD2	CTGATGCCTCTTCCAGGTGTTT	CTGGATGACATATGCCTCCACTCT	AAGCCAGTGTAACAGGATCGCCTATACCACCA
HBD3	CAGAGGCGGCGGTGT	CGAGCACTTGCCGATCTGTT	CTGTGCTCAGCTGCCTTCCAAAGG4
HBD4	GGCAGTCCCATAACCACATATTC	TCCTCTATTAGCCGTTTCTCTT	TGTCCAATTCAAATTCGCTTCTCACTGGA
SLPI	GCATCAAATGCCTGGATCC7	GCATCAAACATTGGCCATAAGTC	TGACACCCAAACCCAACAAGGAGG
Elafin	TGCCTCC TGCCCATTATC	CAGTATCTTTCAAGCAGCGTTAG	ATCCGTTGCGCCATGTTGAATCC

laboratory in sterile phosphate-buffered saline solution (Sigma, Poole, Dorset, UK).

Three endometrial samples were used as positive controls for natural antimicrobials. Because individual natural antimicrobials are expressed differentially throughout the menstrual cycle,[21] an endometrial sample from the period of maximal expression for each natural antimicrobial was used (menstrual sample for elafin and HBD2; proliferative sample for HBD4; mid-secretory sample for HBD1, HBD3, and SLPI). They were collected from women who underwent gynecologic procedures for benign conditions. All of the women had regular menstrual cycles (28–35 days) and had not received any hormonal treatments for 3 months before biopsy collection. Menstrual cycle stage was determined from the date of the patient's last menstrual period, histologic dating, and circulating serum estradiol and progesterone concentrations. Endometrial biopsy specimens were immersed in Tri reagent (Sigma) for RNA extraction. A portion was also fixed in 10% neutral buffered formalin overnight at 4°C, stored in ethanol, and then wax embedded for subsequent histologic examination, which was normal in all cases.

Cell culture

Amnion epithelial cells were isolated with a method adapted from Bennett et al.[22] Amniotic membrane was washed and steeped in EDTA (0.5 mmol/L; Sigma) for 15 minutes. Cells were dissociated by incubation in a solution of Dispase (Gibco, Paisley, UK) for 45 minutes at 37°C, and released by agitation in Roswell Park Memorial Institute 1640 medium (Sigma).

Primary amnion cells were plated in 6-well plates (Nunc; Gibco) at a density of 1.5 to 2×10^6 cells/mL. FL, WISH, and He-La cells (ATCC, Manassas, VA) were plated at a density of 0.5×10^6 cells/mL. Cells were cultured in Roswell Park Memorial Institute 1640 medium that was supplemented with 10% fetal calf serum (Mycoplex; PAA Laboratories, Teddington, UK), penicillin (50 μg/mL; Sigma), streptomycin (50 μg/mL; Sigma) and L-glutamine (2 mmol/L; Sigma) and was maintained at 37°C in 5% Co_2 and 95% air.

After 24 hours of culture, cells were washed, and fresh medium was added. After 72 hours, when confluent, medium was changed to serum-depleted (2% fetal calf serum) for 20 hours before treatments were added; 2% fetal calf serum was used because preliminary experiments showed decreased cell viability after 48 hours of culture in the absence of serum. The epithelial origin of primary cultured cells was confirmed by immunocytochemistry, with >95% of cells positive for the epithelial cell marker pan cytokeratin (Dako, Ely, Cambs, UK).

Treatments

Cells were incubated with recombinant human interleukin (IL)-1β (Peprotech, London, UK) to stimulate natural antimicrobial production. Initial experiments used a dose of 10 ng/mL and treatment times of 6 and 12 hours, because these were the optimal conditions in a previous study.[23] Subsequent experiments investigated the effects of dose or treatment time by using doses of 0.1–100 ng/mL or treatment times of 1, 2, 3, 6, 12, 16, 24, or 48 hours, after which time the effects diminished. All experiments were performed in quadruplicate, with 1 set of duplicates being used to determine mRNA expression by real-time TaqMan quantitative polymerase chain reaction (PCR) and 1 set being used for protein analysis by enzyme-linked immunosorbent assay. Media from the second set of duplicates were stored at −20°C. Treatments had no significant effect on cell numbers or viability at 48 hours, as determined by trypan blue exclusion.

RNA extraction and quantitative PCR

RNA was extracted from amnion with RNeasy minispin columns (Qiagen, Crawley, West Sussex, UK), and from endometrium with the use of Tri-reagent (Sigma), according to manufacturer's protocols. RNA quantity and quality were assessed by the Agilent 2100 bioanalyzer system in combination with RNA_{6000} nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to complementary DNA for real-time PCR.

RNA samples were reverse transcribed with the use of random primers (Taqman RT-PCR kit; Applied Biosystems, Foster City, CA) and amplified by ABI Prism TaqMan 7900 (Applied Biosystems), according to standard protocols. Target mRNA was quantified in relation to 18S ribosomal RNA abundance in each sample. Negative controls were: i) a negative reverse transcribed-sample (RNA template with no reverse transcriptase enzyme), ii) reverse tran-

scribed-H_2O (water in place of RNA template); and iii) a TaqMan-reaction negative control (complementary DNA replaced with H_2O). Positive controls were provided by mRNA that were extracted from endometrium.

Primer/probe combinations were designed with Primer Express software (Applied Biosystems) (Table 1). Sequences were validated by basic local alignment search tool (BLAST) searches to show that the amplified sequence was unique; the linearity of response was checked by the amplification of serially diluted complementary DNA samples and by plotting the corrected values against dilution.

Enzyme-linked immunosorbent assay

HBD2 and SLPI concentrations in cell culture medium were determined with the use of a commercially available HBD2 assay kit (PeproTech) and SLPI assay kit (HyCult, Uden, the Netherlands), according to manufacturer's protocol in 96-well assay plates (Nunc Maxi-sorp; Gibco). An assay for total cellular protein (Bio-Rad Laboratories, Hercules, CA) was performed on the remaining cells, according to manufacturer's protocols. The amount of total cellular protein was used as a denominator for HBD2 and SLPI levels to avoid errors that could result from variation in cell numbers.

Statistical analysis

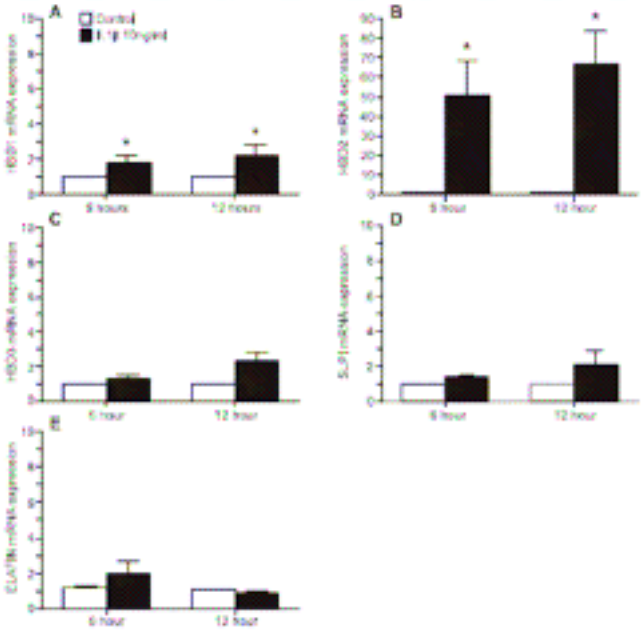
Analysis was carried out with the GraphPad Prism software package (GraphPad Software, Inc, San Diego, CA). Statistical significance was determined by 1-way analysis of variance with Tukey's test to assign individual differences. Nonparametric data were analyzed with the use of the Kruskal-Wallace test. A probability value of $< .05$ was regarded as significant.

RESULTS

Natural antimicrobial mRNA expression by primary cultured amnion epithelial cells

The expression of HBD1, HBD2, HBD3, SLPI, and elafin mRNA was detectable by reverse transcribed quantitative PCR in primary cultured amnion epithelial cells,

FIGURE 1
Natural antimicrobial expression in amniotic epithelial cells



Regulation of natural antimicrobial mRNA expression in primary amnion epithelial cells by IL-1β (10 ng/mL; n = 5). Data are presented as mean ± SEM, relative to untreated controls at 6 and 12 hours. A, HBD1 mRNA expression. The asterisk denotes a probability value of $< .05$ that was determined by analysis of variance. B, HBD2 mRNA expression. The asterisk denotes a probability value of $< .001$ that was determined by analysis of variance. C, HBD3 mRNA expression. D, SLPI mRNA expression. E, Elafin mRNA expression.

whereas HBD4 was undetectable (n = 5). HBD1 mRNA was slightly upregulated by IL-1β 10 ng/mL treatment, which exhibited a 1.81-fold increase that was seen at 6 hours and a 2.24-fold increase that was seen at 12 hours ($P < .05$; Figure 1A). HBD2 mRNA was increased dramatically by IL-1β 10 ng/mL treatment, which exhibited a 51-fold increase at 6 hours and a 67-fold increase at 12 hours ($P < .001$; Figure 1B). Levels of HBD3, SLPI, and elafin mRNA were not affected significantly by IL-1β treatment (Figure 1C-E).

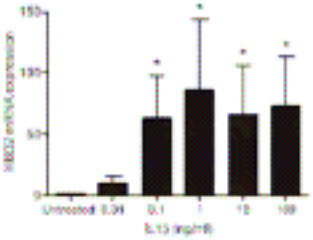
Dose responsive effect of IL-1β on HBD2 production

IL-1β exhibited a dose-responsive effect on HBD2 mRNA that was maximal at 1 ng/mL, which then plateaued ($P < .05$; Figure 2). Subsequent time course experiments used a dose of 10 ng/mL to ensure that sufficient protein was available throughout the whole experiment, despite any possible metabolism.

Pattern of HBD2 mRNA expression in primary cultured amnion epithelial cells

The pattern of HBD2 mRNA expression in response to IL-1β was examined over a 48-hour period in 5 different biologic samples. Treatment significantly upregulated HBD2 mRNA production compared with unstimulated control at all time points that were examined ($P < .05$; Figure 3A). There was no significant change in HBD2 mRNA

FIGURE 2
IL-1β dose response of HBD2



HBD2 mRNA expression IL-1β dose response in primary amnion epithelial cells. Data are presented as mean ± SEM, relative to untreated controls at 6 hours. Cells were treated with IL-1β (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, or 100 ng/mL; n = 3). The asterisk denotes a probability value of < .05 determined by analysis of variance.

expression in unstimulated controls over the 48-hour period (Figure 3B). In all 5 samples, HBD2 mRNA was produced in a biphasic pattern (Figure 3C) with 2 peaks in expression. A typical response from 1 sample is shown, because the periodicity of the response varied between samples; thus the pattern was not as evident when data were averaged. The early response of HBD2 to IL-1β treatment was investigated in 4 samples. This showed that HBD2 mRNA expression became significantly upregulated after 3 hours of treatment with IL-1β 10 ng/mL ($P < .05$; Figure 3D).

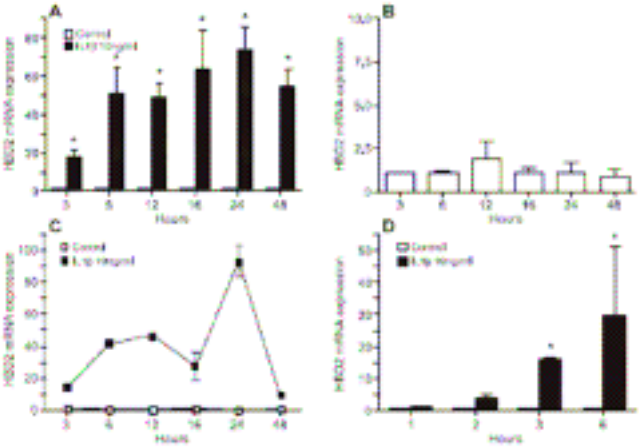
Natural antimicrobial protein expression by primary cultured amnion epithelial cells

HBD2 and SLPI protein levels were measured in cell culture medium by sandwich enzyme-linked immunosorbent assays. Significantly higher levels of HBD2 protein were produced by cells that were treated with IL-1β 10 ng/mL for 24 and 48 hours ($P < .05$; Figure 4). We found no increase in SLPI levels (data not shown).

Natural antimicrobial mRNA expression by cell lines

Natural antimicrobial mRNA expression in primary amnion cells was significantly different from that in FL and WISH cell lines (Table 2). HBD2 mRNA was highly expressed in primary amnion cells but undetectable in FL and WISH cells. HBD1 and HBD3 were expressed at significantly higher levels in primary amnion cells than in FL and WISH cells ($P < .05$). Conversely, SLPI and elafin mRNA were significantly lower in primary amnion cells than in either FL or WISH cells ($P < .05$). Natural antimicrobial mRNA expression in FL and WISH cells was similar to that seen in the He-La cervical cell line that contaminates them, except for the observation that SLPI and elafin mRNA expression was slightly lower in FL cells than in He-La cells ($P < .05$).

FIGURE 3
Pattern of HBD2 mRNA expression in response to IL-1β

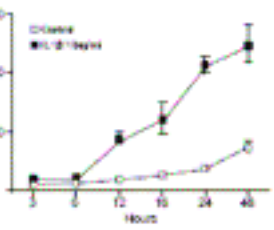


HBD2 mRNA expression in response to IL-1β (10 ng/mL) in primary amnion epithelial cells over time. Data are presented as mean ± SEM. A HBD2 mRNA expression in primary amnion epithelial cells at 3 hours, 6 hours, 12 hours, 16 hours, 24 hours, and 48 hours (n = 5), relative to untreated control at the same time point. B HBD2 mRNA expression in unstimulated primary amnion epithelial cells at 3 hours, 6 hours, 12 hours, 16 hours, 24 hours, and 48 hours (n = 5), relative to 3-hour control. C Typical pattern of HBD2 mRNA expression in primary amnion epithelial cells (n = 1). D HBD2 mRNA expression in primary cultured amnion epithelial cells treated with IL-1β (10 ng/mL) at 1 hour, 2 hours, 3 hours, and 6 hours (n = 4), relative to untreated control at the same time point.

COMMENT

To our knowledge, this is the first study of natural antimicrobial production in primary cultured amnion epithelial cells and demonstrates the expression of HBD1, 2, and 3, SLPI, and elafin. This is consistent with findings in other studies that show HBD1-3, elafin, and SLPI im-

FIGURE 4
HBD2 protein production in response to IL-1β



HBD2 protein expression in response to IL-1β in primary amnion epithelial cells over 48 hours. Data represent mean ± SEM in picograms of HBD2 per milligram total cellular protein (n = 5).

TABLE 2
Comparison of natural antimicrobial mRNA expression in primary amnion cells (n = 5), FL cells (n = 3), WISH cells (n = 3), and He-La cells (n = 3)

Variable	Cells			
	Primary amnion	FL	WISH	He-La
HBD1	5.3 ± 2.0*	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
HBD2	4.0 ± 1.4*	Undetectable	Undetectable	Undetectable
HBD3	9.2 ± 2.9*	1.9 ± 0.6	1.1 ± 0.07	1.8 ± 0.6
SLPI	0.10 ± 0.01*	0.11 ± 0.01†	0.34 ± 0.06	0.20 ± 0.03
Elafin	0.05 ± 0.01*	0.21 ± 0.06‡	0.21 ± 0.06	0.40 ± 0.03

Data are presented as mean ± SEM, relative to the amount in an endometrial positive control.
* $P < .05$ between primary amnion cells and FL, WISH and He-La cells.
† $P < .05$ between FL cells and He-La and WISH cells.
‡ $P < .05$ between FL cells and He-La cells.

munopositivity in the amniotic epithelial layer[10,24,28] and SLPI and elafin mRNA expression in amnion tissue.[20,18]

IL-1β dramatically upregulates the production of HBD2 in primary amnion epithelial cells in a dose and time-dependent manner, which is evidenced at both mRNA and protein levels. Labor is associated with increased numbers of inflammatory cells and production of cytokines such as IL-1β.[1] This suggests that HBD2 production could be increased during parturition, which is a time when the fetus is particularly vulnerable to ascending infection. Because HBD2 is chemotactic for neutrophils[5] and immature dendritic cells and T cells,[6] it could also participate in the recruitment of inflammatory cells that are seen at this time.

Intrauterine infection is associated with an exaggerated inflammatory response and is a major cause of preterm labor.[2] IL-1β is implicated in this process, and women with preterm labor and amniotic infection have elevated amniotic fluid IL-1β concentrations and activity.[25] In this situation, HBD2 production may be upregulated and could act to limit the spread of infection. Because HBD2 mRNA is also stimulated by IL-1β in primary cultured chorion cells,[16] these adjacent tissues may act in tandem.

HBD2 mRNA production in response to IL-1β that showed a biphasic pattern was intriguing. This suggests that there are 2 distinct components to this response. The first peak in production occurs within a few hours and is typical of a rapid innate immune response. The second peak is slower and may represent upregulation that is controlled by a secondary gene product. This requires elucidation, and the production of HBD2 by the fetal membranes in normal and preterm labor settings is under further investigation.

HBD1 mRNA expression in primary amnion epithelial cells was also slightly upregulated by IL1β treatment, although this was only marginal and may not be biologically relevant. Until recently, it was accepted widely that HBD1 was produced constitutively by epithelia, whereas HBD2 was highly inducible by various cytokines, microbes, and bacterial products.[26] New studies, however, have demonstrated that HBD1-4 may all be produced in a constitutive or inducible manner, depending on the site of production and stimulus that is applied.[27] Defensin production in the amnion may also be influenced by different cytokines and inflammatory stimuli.

In this study, IL-1β did not influence amnion epithelial SLPI mRNA. SLPI protein expression was similarly unaffected by IL-1β treatment, which suggests that there is neither de novo production of SLPI nor secretion of preformed protein in response to IL-1β. This differs from findings by Zhang et al[19] that demonstrated an increase in SLPI protein production by primary amnion cells after 48 hours of stimulation with IL-1β. This may reflect differences in culture technique, because we allowed our cells to become confluent before treatment as opposed to stimulating them immediately after dissociation.

We found that IL-1β treatment had no significant effect on the expression of elafin mRNA in amnion epithelial cells, which is in contrast to the stimulatory effect of IL-1β on elafin mRNA production in chorion trophoblast cells.[16] In whole fetal membranes, elafin mRNA expression is decreased in cases of preterm membrane rupture but is increased in cases of chorioamnionitis.[18] Together this evidence suggests that the chorion provides the greater contribution to production of elafin, which may have an important role in the regulation of protease activity within the fetal membranes.

The pattern of natural antimicrobial expression in both FL and WISH cells markedly differed from that in primary cultured amnion epithelial cells. These findings show that cell lines are not representative of amnion and illustrate the care that must be taken when the results are extrapolated from commercial cell lines.

In summary, HBD2 is a potent natural antibiotic that is produced by amnion epithelial cells in response to IL-1β, which also interacts with the adaptive immune system. We believe that it may have an important role in protecting the fetus and could also be an important chemokine that is involved in the onset of parturition. Further study of this response may allow development of new strategies and treatments to help decrease the incidence of premature birth. ■

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REFERENCES
1. Keelan JA, Blumenstein M, Helliwell RJ, Sato TA, Marvin KW, Mitchell MD. Cytokines, prostaglandins and parturition: a review. Placenta 2003;24(suppl A):S33-46.

2. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500-7.
3. Bals R. Epithelial antimicrobial peptides in host defense against infection. Respir Res 2000;1:141-50.
4. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3:710-20.
5. Niyonsaba F, Ogawa H, Nagaoka I. Human beta-defensin-2 functions as a chemotactic agent for tumor necrosis factor-alpha-treated human neutrophils. Immunology 2004;111:273-81.
6. Yang D, Chertov O, Bykovskaia SN, et al. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 1999;286:525-8.
7. Sallenave JM. Antimicrobial activity of antiproteinases. Biochem Soc Trans 2002;30:111-5.
8. King AE, Fleming DC, Critchley HO, Kelly RW. Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol Hum Reprod 2002;8:941-9.
9. Quayle AJ. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. J Reprod Immunol 2002;57:61-79.
10. Zhang Q, Shimoya K, Moriyama A, et al. Production of secretory leukocyte protease inhibitor by human amniotic membranes and regulation of its concentration in amniotic fluid. Mol Hum Reprod 2001;7:673-9.
11. Denison FC, Kelly RW, Calder AA, Riley SC. Secretory leukocyte protease inhibitor concentration increases in amniotic fluid with the onset of labor in women: characterization of sites of release within the uterus. J Endocrinol 1998;161:299-306.
12. Akinbi HT, Narendran V, Pass AK, Markart P, Hoath SB. Host defense proteins in vernix caseosa and amniotic fluid. Am J Obstet Gynecol 2004;191:2090-6.
13. Espinoza J, Chaiworapongsa T, Romero R, et al. Antimicrobial peptides in amniotic fluid: defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. J Matern Fetal Neonatal Med 2003;13:2-21.
14. Feng Y, Pan X, Huang N, Wu Q, Wang B. [The human beta-defensins expression in female genital tract and pregnancy-related tissues]. Sichuan Da Xue Xue Bao Yi Xue Ban 2003;34:212-9.
15. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. FEBS Lett 1996;396:319-22.
16. King AE, Paltoo A, Kelly RW, Sallenave JM, Bocking AD, Challis JR. Expression of natural antimicrobials by human placenta and fetal membranes. Placenta 2007;28:161-9.
17. Buhimschi IA, Jabr M, Buhimschi CS, Petkova AP, Weiner CP, Saed GM. The novel antimicrobial peptide beta3-defensin is produced by the amnion: a possible role of the fetal membranes in innate immunity of the amniotic cavity. Am J Obstet Gynecol 2004;191:1678-87.
18. Tromp G, Kuivaniemi H, Romero R, et al. Genome-wide expression profiling of fetal membranes reveals a deficient expression of proteinase inhibitor 3 in premature rupture of membranes. Am J Obstet Gynecol 2004;191:1351-8.
19. Nelson-Rees WA, Flandermeyer RR. HeLa cultures defined. Science 1976;191:96-8.
20. Kniss DA, Xie Y, Li Y, et al. ED(27) trophoblast-like cells isolated from first-trimester chorionic villi are genetically identical to HeLa cells yet exhibit a distinct phenotype. Placenta 2002;23:32-43.
21. King AE, Critchley HO, Kelly RW. Innate immune defenses in the human endometrium. Reprod Biol Endocrinol 2003;1:116-24.
22. Bennett PR, Rose MP, Myatt L, Elder MG. Preterm labor: stimulation of arachidonic acid metabolism in human amnion cells by bacterial products. Am J Obstet Gynecol 1992;166:649-55.
23. McDermott AM, Redfern RL, Zhang B, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. Invest Ophthalmol Vis Sci 2003;44:1659-65.
24. Buhimschi IA, Jabr M, Buhimschi CS, Petkova AP, Weiner CP, Saed GM. The novel antimicrobial peptide beta3-defensin is produced by the amnion: a possible role of the fetal membranes in innate immunity of the amniotic cavity. Am J Obstet Gynecol 2004;191:1678-87.
25. Romero R, Mazor M, Brandt F, et al. Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. Am J Reprod Immunol 1992;27:117-23.
26. Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA. Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. Infect Immunol 1998;66:4222-8.
27. Joly S, Organ CC, Johnson GK, McCray PB Jr, Guthmiller JM. Correlation between beta-defensin expression and induction profiles in gingival keratinocytes. Mol Immunol 2005;42:1073-84.

Appendix 6: Microarray Data (CD)